DOI: 10.1111/iep.12294

WILEY INTERNATIONAL JOURNAL OF Experimental Pathology

Matrix Biology Europe – July 2018 Meeting

Celebrating 50 years of Federation of European Connective Tissue Societies Meetings

(organised by the British Society for Matrix Biology and the University of Manchester Wellcome Trust Centre for Cell-Matrix Research)

The effect of extracellular matrix stiffness on pancreatic cancer stem cells in a 3D matrix model

Y. Liu*,†; B. O. Okesola†; O. M. Pearce*; A. Mata†; C. Heeschen*

*Barts Cancer Institute, Queen Mary University of London, London, United Kingdom; †School of Engineering and Materials Science, Queen Mary University of London, London, United Kingdom

Introduction: Pancreatic cancer is a CR-UK cancer of unmet need, primarily due to delayed diagnosis and ineffective treatments. The marked fibrosis and dense stroma is thought to serve as a barrier to anticancer drugs in pancreatic ductal adenocarcinoma (PDAC). As the rigid matrix forces and counteracts the cancer cells, the stiffened extracellular matrix (ECM) can affect cellular behavior and promote tumour development. Cancer stem cells (CSCs), a small percentage of the cancer cell population, are characterized by their multipotency and the ability to initiate cancer, propagate metastases, and are resistant to chemotherapeutic drugs. We hypothesized that the stiffened ECM may support CSCs, and therefore promote PDAC development. Therefore, we employed a self-assembling peptide amphiphile (PA) that exhibits tuneable rigidity as a 3D matrix model to investigate the relationship between stiffness ECM and CSCs in pancreatic cancer progression.

Materials and Methods: A new PA was designed in our group, and different stiffness of PA gel was obtained by introducing different concentrations of $CaCl_2$. AFM was applied to measure the stiffness of pancreatic cancer patient-derived xenografts (PDX); confocal imaging and q-PCR were used to examine cell viability, EMT and CSC gene expression.

Results: The stiffness range of PDX is about 1–20 kPa; the corresponding stiffness PA gel was formed by 0.01–0.1 M $CaCl_2$. Compared to 2D cell culture, PDAC in PA hydrogel showed good cell viability within 21 days. As the stiffness of hydrogel increased, the EMT- and CSC-related gene expression in PDAC increased at mRNA level; CSC gene expression elevated more once hyaluronan was introduced into stiffer hydrogel; PDAC encapsulated in stiffer hydrogel showed the high chemotherapy resistance.

Discussion: These data show that stiffer ECM stimulates EMT- and CSC-related gene expression and decreases antineoplastic drug sensitivity in PDAC. Many studies reported that cells respond to force on integrin-mediated adhesions by remodeling the ECM through upregulating FAK and PI3K, and activating Rock to increase actomyosin-mediated cellular tension. Therefore, future studies will focus on FAK/PI3K/Rac1 or Rock signal pathway to investigate the relationship between stiffness matrix and pancreatic CSCs. This study highlights the potential for targeting CSCs via mechanical property of tumour microenvironment as promising therapeutic strategy that inhibit tumour progression.

Sirt1 Activation in ESC-derived prechondrocytes promotes cartilage ECM expression

C. A. Smith*; M. Dvir-Ginzberg†; S. J. Kimber*

*University of Manchester, United Kingdom; †HUJI, Israel

Introduction: Regulation of gene expression and transcription factors by epigenetic factors is essential for successful differentiation. SIRT1 is a histone deacetylase enzyme, able to bind and deacetylate the main chondrogenic factor SOX9. Indeed, osteoarthritic and dedifferentiating primary chondrocytes display decreased SIRT1 protein expression. The aim of this study is to identify the role and activity of SIRT1 during the differentiation of human pluripotent stem cells hPSCs to chondrocytes and its impact of extracellular matrix expression.

Materials and Methods: hPSCs were differentiated to prechondrogenic cells using a 2D 14-day defined differentiation protocol. At 14 days, cells were pelleted and cultured for an additional 14-day period in 3D pellet culture, with SIRT1 activator (SRT1720) or inhibitor (EX527). Additionally, TC28a2 immortalized juvenile chondrocytes were cultured in pellet culture, with SIRT1 activator or inhibitor. qRT-PCR and protein expression were used to assess chondrogenic output. ChIP-PCR was used to determine chromatin binding of SIRT1 under different conditions. Histological assessment was performed to determine pellet structure.

Results: Results show no beneficial effect of activation or inhibition of SIRT1 during the 2D chondrogenesis stage with no change to COL2A1 or ACAN gene expression. During 3D culture, inhibition of SIRT1 caused no significant change in gene expression compared to control. Activation of SIRT1 in 3D led to significant increases in SOX5, ARID5B and ACAN, with significant decreases in COL1A1 and RUNX2 gene expression (Fig 1). Likewise, activation of SIRT1 in TC28a2 cells only led to an increase in ECM gene expression in 3D not 2D, in particular SOX5 and ACAN. This was supported by Western blot analysis of ACAN which showed a 3.5-fold increase in activated cells. Overexpression of SIRT1 in TC28a2 cells did not result in an increase of ECM gene expression.

Discussion: The results of this study indicate that SIRT1 expression and activity are important to PSC-derived chondrocyte development, by being involved with a protein complex required for the transcription of chondrogenic genes.

Identification of synovial fluid proteins that are associated with early osteoarthritis treatment failure: the search for novel markers leads us back to matrix metalloproteinases (MMPs)

C. H. Hulme[*,†]; E. Wilson[†,‡]; H. R. Fuller[*]; S. Roberts[*,†]; J. B. Richardson[*,†]; P. Gallacher[*,†]; M. J. Peffers[§]; S. L. Shirran[¶]; C. H. Botting[¶]; K. T. Wright[*,†]

[*]Institute of Science and Technology in Medicine, Keele University, Keele, Staffordshire, United Kingdom; [†]Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, United Kingdom; [‡]Chester Medical School, Chester University, Chester, United Kingdom; [§]Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, United Kingdom; [¶]BSRC Mass Spectrometry and Proteomics Facility, University of St Andrews, North Haugh, Fife, KY16 9ST, United Kingdom

Introduction: Autologous chondrocyte implantation (ACI) is a cell therapy used to treat cartilage defects and early osteoarthritis. During initial surgery (Stage I), healthy cartilage is harvested from the joint. Chondrocytes are isolated and culture-expanded before being implanted into the defect site (Stage II). An alternative cell-based therapy uses patients' bone marrow (BM), which is collected without cartilage harvest at Stage I; subsequently culture-expanded BM-mesenchymal stromal cells (MSCs) are implanted at Stage II. 20% of ACI-treated patients demonstrate no clinical benefit. We aim to better understand why these individuals fail.

Materials and Methods: Two proteomic techniques (isobaric tag for relative and absolute quantitation and label-free quantitative liquid chromatography tandem mass spectrometry) were used to comprehensively profile the proteome of knee synovial fluid (SF) samples. Fourteen ACI responders' and 13 non-responders' SFs collected at Stages I and II were analysed. ACI response was determined by change in functional knee score at 12 months. Proteins showing differential levels were validated using Quantikine® immunoassays in this cohort and a further independent group of patients which included those treated with BM-MSCs (ie, no cartilage harvest procedure).

Results: Proteomic analyses highlighted that MMPs 1 and 3 demonstrate increased abundance at Stage II compared to Stage I, only in the ACI failure group (MMP-1 increased 2.7-fold; MMP-3 increased 2.9-fold). These observations were validated by immunoassay (MMP-1, Stage I: 800 ± 889 pg/mL, Stage II: 7741 ± 8065 pg/mL, $P = 0.006$; MMP-3, Stage I: 34 ± 17 ng/mL, Stage II: 61 ± 17 ng/mL, $P = 0.002$, Mann-Whitney U test). In the independent cohort, MMP-1 ($n = 31$) and MMP-3 ($n = 47$) were increased at Stage II compared to Stage I, only in the patients who underwent a cartilage harvest procedure during Stage I ($P < 0.0001$; Wilcoxon matched pairs).

Discussion: This study suggests that there may be an acute catabolic response in the knee to the cartilage harvest procedure in ACI only in individuals who do not respond well clinically. Further study is required to determine whether the cartilage harvest procedure itself is damaging in these patients or whether this response to cartilage injury is indicative of a poor innate capacity for cartilage repair.

Insulin-like growth factor binding protein (Igfbp6) is a cross-species transcriptomic tendon marker

A. J. Mueller-Breckenridge; A. J. Turlo; D. Zamboulis; M. J. Peffers; P. D. Clegg

Department of Musculoskeletal Biology I, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool L7 8TX, United Kingdom

Introduction: Evaluation criteria of bioengineered tendon constructs are unclear partially due to the lack of specific markers of tendon development and differentiation. The aim of this study was to identify a panel of genes that exhibit clearly higher expression in tendon relative to cartilage and muscle and validate them across key model species utilised in tendon research.

Materials and Methods: Comprehensive gene expression profiling of rat tendon and cartilage was undertaken using two independent microarray platforms. Illumina RatRef v12 was used for analysing whole tissues while Affymetrix GeneST Rat for isolated primary tenocytes and chondrocytes. Processing of raw gene expression data and differential expression analysis was undertaken using software packages in R. Genes that demonstrated high correlation in expression levels across two studies were validated by qRT-PCR in whole rat tendon relative to cartilage and muscle. Five

genes demonstrating the highest expression in validation experiment were selected for further evaluation by qRT-PCR across different musculoskeletal tissues in ovine and equine.

Results: Genes that demonstrated the highest tendon expression (log2 fold change > 1.5) in both microarray studies, relative to cartilage, included *Tmnd*, *Serpinf1*, *Igfbp6*, *Cxcl13*, *Cpxm2*, *Mfap5* and *Aspn*. *Meox2*, *Mustn1*, *Thbs4*, *Thbs2* and *Prrx1* demonstrated more variable expression between the two platforms. Genes showing higher expression in tendon were enriched for functional terms relating to "developmental processes" and "extracellular matrix." In qRT-PCR analysis of rat musculoskeletal tissues, significantly higher expression in tendon was detected for *Cpxm2*, *Myoc*, *Mfap5*, *Serpf1* ($P < 0.05$) and *Aspn*, *Ecm1*, *Igfbp6*, *Tnmd* and *Thbs4* ($P < 0.001$). Only *Igfbp6* and *Tnmd* demonstrated significantly higher expression in the tendon of all species relative to cartilage and muscle.

Discussion: The initial pool of tendon gene markers, identified by unbiased transcriptomic analysis of musculoskeletal tissues in rat, demonstrated high variability in other model species. Insulin-like growth factor binding protein 6 (*Igfbp6*) was identified as the only universal tendon marker, comparable with that previously recognised, tenomodulin (*Tnmd*). Altered expression of *Igfbp6* has been described previously in animal models of tendon injury and human fibroblasts affected by Dupuytren's disease. *Igfbp6* may be considered a potential reference biomarker for evaluation of tendon physiological function and directed development of engineered tendon.

Matrix-inspired biomaterials for cell phenotype control

J. L. Thompson[*,†]; S. Pijuan-Galitó[*,†]; J. C. Ashworth[*,‡]; K. S. Dowding[*]; A. Nasir[*,†]; J. G. W. Smith[*]; T. Wilpshaar[§]; J. Bovée[§]; J. Liu[¶]; C. Denning[*]; M. R. Alexander[†]; C. L. R. Merry[*]

Division of Cancer and Stem Cells; †School of Pharmacy, University of Nottingham, United Kingdom; ‡University of Manchester, United Kingdom; §Leiden University Medical Centre, The Netherlands; ¶University of North Carolina, USA

Introduction: Heparan sulphate (HS) glycosaminoglycans (GAG) found on the cell surface or in the ECM bind a multitude of ligands to influence cell proliferation, differentiation, adhesion and migration. There are currently a few, rare disorders linked to mutations in HS genes, such as multiple osteochondroma (MO), with tissue-specific phenotypes that are puzzling given the ubiquitous expression of HS. Complex *in vivo* and *in vitro* assay systems currently impede our ability to define the specific mechanistic basis of ECM regulation in development and disease, critical for understanding matrix control of cell behaviour. To address the need for defined culture environments with which to model the ECM, and ECM-related diseases, we will assess the impact of matrix components on directed stem cell differentiation using a synthetic self-assembling peptide hydrogel-based 3D culture method.

Materials and Methods: Human induced pluripotent stem cells (hiPSCs) were cultured in non-functionalised self-assembling octapeptide hydrogels under serum-free pluripotency-supporting and differentiation (spontaneous and neural) conditions. Immunocytochemistry was used to visualise protein and GAG expression in the gels. 2D screening of synthetic GAG oligos with defined compositions was used to identify GAG structures best able to support hiPSC differentiation. To create a disease model, hiPSCs were reprogrammed from MO patient material.

Results: Early data demonstrate continuous culture and differentiation of hiPSCs in "naked" unmodified hydrogels, in the absence of any exogenous matrix components (eg, Matrigel, serum or co-culture). 2D screening shows GAG structures with slight variations in sulphation patterns differentially regulate neural differentiation of hiPSCs. hiPSCs generated from an MO patient exhibit a normal karyotype and show differences in heparan sulphate expression compared to WT controls.

Discussion: We can use this 3D culture system to assess the impact of matrix additions (eg, synthetic GAGs shown to have bioactivity from 2D screens) as well as rheological mechanics, in a fully controlled 3D manner. We have described for the first time reprogramming of MO patient material into hiPSCs. CRISPR/Cas9 gene editing will be used to generate MO patient isogenic controls prior to encapsulation and study in the gels.

Comparison of chondrogenic potential of bone marrow- and umbilical cord blood-derived mesenchymal stem cells in an attempt to improve cartilage tissue engineering strategies

R. Contentin[*,1]; T. Branly[*,1]; J. Pestel[*]; M. Concari[*]; M. Desancé[*]; S. Jacquet[†]; L. Bertoni[†]; F. Audigié; [*] M. Demoor[*]; P. Galéra[*]

Normandie Univ, Unicaen, Biotargen, 14000 Caen, France; †Center of Imaging and Research on Locomotor Affections in Equines, Ecole Vétérinaire d'Alfort, Université Paris-Est, 14430 Goustranville, France

Introduction: Cartilage tissue engineering and/or cell therapy offer new insights to cure articular disorders such as osteoarthritis. Mesenchymal stem cells (MSC) represent an attractive cell type in order to produce a hyaline cartilage substitute. This study aimed to compare the chondrogenic

potential of equine umbilical cord blood (UCB)- and bone marrow (BM)-derived MSC.

Materials and Methods: BM and UCB MSC were isolated and then amplified in monolayer culture. We characterized MSC by assessing their proliferative and multipotency capacities, and the presence of cluster of differentiation characteristics of MSC. Then, we compared MSC at their undifferentiated basal state and after chondrogenic differentiation. Chondrogenesis was induced by culturing the cells in a type I/III collagen biomaterial with BMP-2 and TGF-ß1. To determine the best oxic condition, the chondrogenic differentiation was performed either in hypoxia and normoxia. To compare the MSC chondrogenic potential, we evaluated the mRNA levels and protein amounts of several osteogenic (osteocalcin/Runx2), hypertrophic (type X collagen), fibrocartilage (type I collagen) and chondrogenic markers (type II collagen). Furthermore, the extracellular matrix (ECM) composition/structure was analysed by immunohistochemistry.

Results: The MSC characterization allowed to determine that UCB and BM MSC have different multipotency capacities as well as proliferative abilities. At their undifferentiated basal status, UCB and BM MSC exhibited also differences in the expression of type I collagen and of osteogenic, chondrogenic, hypertrophic markers. Upon culture of the cells in a chondrogenic induction medium, both MSC were able to increase their chondrogenic markers' expression and seemed to produce an ECM of better quality in hypoxia, although type I collagen remained expressed. Nevertheless, both MSC exhibited an upregulation of type I collagen synthesis. UCB MSC produced the highest protein amounts of type II collagen but also of type I collagen, whatever the oxic condition.

Discussion: Both BM and UCB MSC were able to produce a hyaline-like cartilage matrix upon a chondrogenic differentiation protocol whatever the oxic condition. However, considering that type I collagen is the major undesirable component in the *in vitro* cartilage neosynthesis, BM MSC seem to be the best MSC candidate for cartilage tissue engineering.

[1]Contributed equally.

In vivo wound healing with an engineered matrix-rich living modular construct for stem cell delivery

K. Spanoudes*,†; D. Gaspar*,†; S. Korntner*,†; Y. Bayon‡; D. I. Zeugolis*,†

Regenerative, Modular & Developmental Engineering Lab (REMODEL), National University of Ireland, Galway, Ireland; †Centre for Research for Medical Devices (CURAM), National University of Ireland, Galway, Ireland; ‡Sofradim Production, Trevoux, France

Introduction: Advanced therapeutic medicinal products for engineering cutaneous substitutes are an evolving option, aiming to improve wound healing outcomes by reducing wound healing closure time and delivering cells, medical compounds and biologics. Matrix-rich tissue equivalents can be fabricated in vitro by employing biophysical, biological and biochemical cues. Our work is focused on the production of matrix-rich implantable tissue equivalents. Herein, we investigated the effect of macromolecular crowding on the fabrication of a matrix rich tissue equivalent for a wound healing application.

Materials and Methods: A collagen-based film has been utilised for the fabrication of a modular, matrix-rich cell carrier (bone marrow stem cells) for wound healing. The structural, mechanical and thermal properties of the material were assessed with electronic microscopy, uniaxial mechanical testing and differential scanning calorimetry (DSC) respectively. For the enhancement of extracellular matrix deposition, a macromolecular crowding agent (Carrageenan) was utilised at all time points. Matrix deposition was assessed with immunocytochemistry. A splinted wound healing model in athymic nude mice was utilised to assess wound healing in vivo. Wound healing closure ratio was assessed on days 3, 7 and 14. Tissues were harvested 14 days postimplantation for histology.

Results: Extracellular matrix deposition has been enhanced at all time points when carrageenan was used as a macromolecular crowding agent in the in vitro regime. Constructs grown in vitro in the presence of carrageenan facilitate accelerated and improved wound healing in vivo when implanted in the athymic nude mouse model.

Discussion: Macromolecular crowding enhances matrix deposition in vitro, contributing to the *in vitro* maturation of implantable modular constructs which translates to better and faster wound healing *in vivo.*

Deriving nucleus pulposus-like progenitors from MAP kinase interference-coupled chondrogenic induction in mesenchymal stem cells

W. K. Tam*; D. Sakai†; D. Liu*; J. Lee*; K. M. C. Cheung*; R. S. Tuan‡; V. Leung*

Department of Orthopaedics and Traumatology, HKU, HK; †Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Japan; ‡Center for Cellular and Molecular Engineering, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction: Progenitor cells expressing tyrosine kinase receptor 2 (Tie2) and disialoganglioside 2 (GD2) were identified in nucleus pulposus (NP) tissue with clonogenicity and self-renewal ability, suggesting regenerative capacities for intervertebral disc (IVD) disease. By microarray transcriptomic analysis, *Cadherin 2* (*Cdh2*), *keratin 19* (*Krt19*) and *carbonic anhydrase 3* (*Car3*) were over-presented in rat primitive NP cells (PNPCs)

versus chondrocytes. *In silico* analysis of signalling pathways suggested PNPCs exhibit low MAP kinase activities. We hypothesized that MAP kinase interference could induce mesenchymal stem cells (MSCs) into NP progenitor-like state. Firstly, we characterized protein expressions of molecular markers in colony-forming units-spherical (CFU-S) from human NP cells. Thereafter, we evaluated gene expressions of CFU-S molecular markers in MAP kinase interfered MSC micro-pellets.

Materials and Methods: CFU-S assay was accessed by seeding 1×10^3 human NP cells/1 mL of methylcellulose medium (*Stem Cell Technologies*) for 14 days and immunostained with (*Santa Cruz*) anti-Tie2, anti-Krt19 and anti-Car3; (*Abcam*) anti-Cdh2, anti-CD24, anti-type II collagen (COL2) and anti-aggrecan (AGC1); and (*BD Biosciences*) anti-GD2 antibodies. TGF-β1 chondrogenic-induced human bone marrow MSC micro-pellet culture was treated with/without 10 ng/mL MEK 1/2 inhibitors (PD98059) for 14 days. Quantitative PCR (qPCR) was performed using TaqMan probes (*Thermo Fisher Scientific*): *TEK (*HS00945746_m1), *CDH2* (HS00983056_m1) and *AGC1* (HS00153936_m1); and normalized by *GAPDH* (HS02758991_g1).

Results: By immunostaining, Tie2, GD2, CDH2, AGC1 and COL2 were detected in NP-derived CFU-S. qPCR showed strong upregulations of *TEK1* and *CDH2* at Day 1 and *AGC1* at Day 14 in MEK1/2-inhibited MSC micro-pellets with robust proteoglycan deposition.

Discussion: MAP kinase interfered MSC micro-pellets could induce CFU-S molecular markers: *Tie2*, *CDH2* and *AGC1* with enhanced proteoglycan deposition, which provides an effective protocol of deriving NP progenitors for IVD engineering.

Identification and *in vitro* screening of osteogenic metabolites through supplement-free nanovibration-driven mesenchymal stem cell differentiation

T. Hodgkinson*; K. Burgess†; D. France‡; P. Campsie§; S. Robertson§; D. Phillips§; P. Childs*; R. O. C. Oreffo¶; S. Reid‡; M. Sanchez-Salmeron*; M. J. Dalby*

*Centre for the Cellular Microenvironment, College of Medical, Veterinary and Life Sciences, University of Glasgow, Scotland; †Glasgow Polyomics, University of Glasgow, Scotland; ‡School of Chemistry, University of Glasgow, Scotland; §SUPA, University of Glasgow, Scotland; ¶Centre for Human Development, Stem Cells and Regeneration, University of Southampton

Introduction: There is a need for the development of effective tissue engineering approaches to produce bone. In the laboratory, these approaches typically involve osteogenic differentiation of mesenchymal stem cells (MSCs) through medium supplementation. We recently developed a supplement-free osteogenic differentiation protocol through nanovibrational stimulation of MSCs[1]. Here, we hypothesised that nanovibrational differentiation of MSCs would allow metabolomic analysis of differentiation without confounding exogenous medium supplements. We aimed to investigate MSC nanovibration-driven osteogenesis in 2D and 3D cultures, identify key osteogenic metabolites and metabolomic processes and investigate their osteogenic potential by supplementing these pathways *in vitro*.

Materials and Methods: Human MSCs were cultured in standard tissue culture well plates (2D) or in type I collagen gels (3D) and cultured over 28 days in three groups—nanovibrational stimulation, osteogenic media (dexamethasone) and MSC expansion media. Differentiation was tracked through changes in gene expression (qPCR) and protein expression (immunofluorescent staining (IFS)). At key points, cell metabolomic analysis was performed (LC-MS; ZIC-pHILIC). We selected the most promising metabolite during osteogenic differentiation. This was synthesised along with several chemical analogs. The osteogenic potential of these metabolites was then investigated through gene and protein expression following supplementation to 2D and 3D MSC cultures.

Results: Nanovibration upregulated key osteogenic genes in both 2D and 3D cultures comparably to osteogenic media, including early upregulation of RUNX2 ($2D \times 14.5$, $P < 0.05$; $3D \times 11.5$, $P < 0.05$) followed by maturation marker osteopontin ($2D \times 19$, $P < 0.05$; $3D \times 7.2$, $P < 0.05$). Corresponding increases in osteogenic proteins were also observed. Metabolomic analysis identified several key networks, with cholesterol sulphate (CS) identified as a promising metabolite target. When CS and several analogs were supplemented at 1 μM to 2D and 3D cultures, they induced osteogenic gene and protein expression, comparably to osteogenic media, while having less off-target effects. In particular, fludrocortisone and fludrocortisone acetate significantly increased osteogenic marker expression, even versus osteogenic media.

Discussion: Nanovibration is an exciting tool for the supplement-free study of MSC osteogenic differentiation, while this work also validates a targeted metabolite supplementation approach for controlling cell fate decisions, which may prove cheaper and more specific than conventional approaches.

Investigating the shared molecular basis of diseases of glycosaminoglycan synthesis and degradation

K. Dowding*; J. Thompson*,†; J. Ashworth*; L. Kjellén‡; J. Smith*; C. Denning*; C. Merry*

*Division of Cancer and Stem Cells, University of Nottingham, United Kingdom; †School of Pharmacy, University of Nottingham, United Kingdom; ‡Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden

Introduction: Glycosaminoglycans (GAGs), including heparan sulphate (HS), are involved in the regulation of many

essential processes such as cell survival, migration, differentiation and adhesion via regulation of multiple interacting signalling pathways. Mutations in genes for key enzymes in the HS synthetic and degradative pathways, such as IDUA, EXT1 and NDST1, lead to severe pathologies, including mucopolysaccharidosis type I (MPSI), multiple osteochondromas (MO) and autosomal recessive intellectual disability (ARID) respectively. Although the phenotypes of these diseases are different, they share a characteristic accumulation of abnormal GAG structures which then impact on the GAG-regulated pathways introduced above. We are creating induced pluripotent stem cell (iPSC)-based disease models, either by reprogramming patient tissue or by CRISPR-Cas9 knockout. Critically, as these disorders are associated with altered pericellular matrix deposition, we will use a defined, fully synthetic peptide hydrogel system, free from exogenous GAG, to study 3D iPSC growth and differentiation.

Materials and Methods: Human iPSCs are cultured in non-functionalised self-assembling peptide hydrogels in Essential 8 (E8) and E6 media. Immunocytochemistry and flow cytometry will be used to characterise the GAG profiles of the different cell lines. N2B27 media will be used to direct neural differentiation. Human iPSCs will be transfected with wild-type CRISPR Cas9 constructs to knock out EXT1, NDST1 and IDUA, creating both heterozygous and homozygous cell lines.

Results: Successful culture and differentiation of human wild-type iPSCs in the peptide hydrogels has been achieved, without the need for serum, other matrix addition or co-culture. Immunocytochemical staining of GAG chains in 2D and 3D culture using specific antibodies has proved to be an effective method to visualise the GAGs deposited by the cells. Guide and targeting plasmids for CRISPR Cas9 gene editing have been constructed ready for transfection.

Discussion: Culturing cells in a defined, GAG-free 3D system allows the study of disease-typical GAGs secreted by cells engineered to model matrix-relevant genetic diseases. The system also allows for the addition of defined ECM components to test their impact on cell behaviour. We therefore aim to develop a suite of models to enable us to study the potential shared mechanistic dysregulation of signalling pathways between diseases of GAG synthesis and GAG degradation.

Nanotopography of substrates directs the deposition of organised fibrillar collagen by corneal stromal cells

D. A. O'Loughlin*,†; H. J. Levis*; V. R. Kearns*; C. Sheridan*; E. G. Canty-Laird†

**Department of Eye and Vision Science, Institute of Ageing and Chronic Disease, University of Liverpool, United Kingdom; †Department of Musculoskeletal Biology I, Institute of Ageing and Chronic Disease, University of Liverpool, United Kingdom*

Introduction: The corneal stroma constitutes 90% of the cornea. It consists of around 200 collagen lamellae, oriented roughly orthogonally throughout. Within each of these are highly aligned collagen fibrils interspersed with keratocytes which are responsible for maintaining transparency. Corneal scarring is a leading cause of blindness globally. A shortage of donors, with only 1 cornea available for every 70 corneas needed, has led to a tissue engineering solution being required. Furthermore, current *in vitro* models of the stroma focus on using a collagen gel or layers of fibroblasts that fail to replicate the microstructure. By being able to more faithfully recapitulate the stroma, we aim to develop an *in vitro* model to better explore the mechanisms of collagen alignment.

Materials and Methods: Cells isolated from human corneoscleral rims were cultured on coverslips with polytetrafluoroethylene (PTFE) nanofibres. Cell Tracker and a collagen probe (CNA35) were added to culture media and imaged for up to 3 days to observe the live cell deposition of collagen. Separate samples were cultured for several weeks to form a cell layer, and the alignment of the extracellular matrix (ECM) was analysed using OrientationJ, an ImageJ plugin.

Results: Collagen fibres could be visualised using a FITC-labelled collagen probe (CNA35) whilst cells were highlighted using Cell Tracker. OrientationJ analysis of collagen fibres showed that when the cells were cultured on PTFE nanofibres, around 73% of the fibrils were aligned within ± 10° of the dominant direction, compared to just 44% when cultured on a control substrate.

Discussion: It has been well documented that cells respond to topographical cues and this has been exploited in a variety of applications. The PTFE nanofibres provided the topographical cues for cells, and thus collagen, to align along. Interestingly, we observed that as stromal cells stratified, they maintained their alignment but rotated by 37°. Literature suggests that cell layers that maintain organisation have improved tissue functionality. By manipulating cell sheets, we hypothesise that we will be able to produce multiple cell layers that can be organised to recapitulate the native cornea for use as either a graft alternative or an *in vitro* model.

Synthetic photoreceptor engineering for optogenetic control of TGFβ signalling

P. Humphreys; C. Smith; R. Lucas; S. Kimber

The University of Manchester

Introduction: The transforming growth factor-β (TGFβ) superfamily (including bone morphogenetic proteins—BMPs) are a family of signalling molecules crucial in chondrogenic development. Current chondrogenic directed differentiation

protocols (DDPs) of human pluripotent stem cells (hPSCs) rely upon timed supplementation of growth factors. However, this may lead to poor differentiation reproducibly and quality because of batch-to-batch variation. Due to the essential role of the TGFβ family during chondrogenesis, precise control of the signalling pathway may enable refinement of differentiation and improvement of ECM production. The aim of this project is to utilise the novel technology of optogenetics to render the activation of the BMP pathway light-sensitive, enabling fine-tuning of signalling during chondrogenesis.

Materials and Methods: Differentiation of MAN13 human embryonic stem cells (hESCs) towards chondrocytes was performed following an established protocol. Differentiation was evaluated through RT-qPCR gene expression analysis of key chondrogenic markers. Optogenetic BMP-like receptors were generated through PCR and "NeB HiFi Assembly" cloning and inserted into a doxycycline inducible vector. Chondrosarcoma SW1353 cells were transduced with lentiviral particles containing a BMP-like SMAD1/5/8 transcriptional response element (BRE) reporter along with optogenetic BMP Type I and II receptors. Cells were dosed with doxycycline before flashing with blue light and SMAD1/5/8 transcriptional activity was measured through nano-luciferase production.

Results: An established chondrogenic DDP resulted in significant upregulation of chondrogenic-associated gene expression SOX9, SOX5 and COL2a1 after 14 days in 2D monolayer culture. Optogenetic BMP-like receptors were successfully generated and expressed by transduced cells through stimulation with doxycycline. Activation of optogenetic receptors with blue light resulted in nano-luciferase production, indicating stimulation of the BRE reporter by transcriptionally active BMP-like SMAD1/5/8.

Discussion: Findings shown here demonstrate the applicability of optogenetics for control of BMP signalling and a framework for future approaches. Light-stimulated dimerisation of Type I and II BMP-like receptors appeared sufficient to stimulate SMAD1/5/8 activity, and variable light dosage should enable fine-tuning of signal transduction. Incorporation of light-controlled BMP signalling in chondrogenic directed differentiation of hESCs may reveal rate-limiting steps and enable improvement of chondrogenic differentiation in the future.

Manipulating co-regulators of RUNX2 and SOX9 to enhance the chondrogenic potential of chondrogenic progenitor cells in osteoarthritis

J. Janßen*; N. Miosge*; B. Schminke*; V. Rosen†; E. Monsonego-Ornan‡

*Tissue Regeneration Work Group, Georg August University, Goettingen, Germany; †Developmental Biology, Harvard Dental School, Boston, USA; ‡Biochemistry, Hebrew University, Rehovot, Israel

Introduction: The regeneration of diseased hyaline cartilage continues to be a great challenge, mainly because degeneration overtaxes the tissue's self-renewal capacity. Recently, we demonstrated that repair tissue from human articular cartilage during the late stages of osteoarthritis harbors a unique progenitor cell population, termed chondrogenic progenitor cells (CPCs). Down-regulation of the osteogenic transcription factor RUNX2 enhanced the expression of the chondrogenic transcription factor SOX9. This, in turn, increased the matrix synthesis potential of the CPCs without altering their migratory capacity. We now present unpublished data on the role of co-regulators of SOX9 and RUNX2 to enhance the chondrogenic potential of CPCs.

Materials and Methods: Candidate molecules, for example, RAB5C, YWHAE or DDX5, identified in an elaborated knockdown and pull-down experiment have been overexpressed or knocked out via CRISPR/Cas9. The effect on the chondrogenic potential was investigated by qPCR, Western blot and immunohistochemistry.

Results: The manipulation of co-regulators of SOX9 and RUNX2 enhances the chondrogenic potential of CPCs. We observed altered expression levels of RUNX2, SOX9 and collagen type I and type II in 2D and 3D cell culture.

Discussion: Our data indicate that we can successfully manipulate the chondrogenic potential of CPCs in vitro. Further research will focus on the improvement of the cartilage composition in vivo, by transplantation of suitable candidate cell lines into the mouse model. This will lead to the identification of new signalling pathways as attractive targets for future OA therapy.

Altered matrix adhesion, impaired function and mitochondrial hyperactivity in endothelial colony-forming cells isolated from patients with diabetic foot ulcers

A. W. W. Langford-Smith*; M. Floren†; A. Hasan‡,§; R. Weston*; N. Edwards*; S. T. Rashid‡,¶,**; A. Boulton‡,*; F. Bowling‡,¶,*; W. Tan†; F. L. Wilkinson*; M. Y. Alexander*,**

*Cardiovascular Science, The Centre for Bioscience, Faculty of Science and Engineering, Manchester Metropolitan University, United Kingdom; †Mechanical Engineering, University of Colorado Boulder, USA; ‡Diabetes Research Group, University of Manchester, United Kingdom; §Universiti Kuala Lumpur, Malaysia; ¶Department of Vascular and Endovascular Surgery, Manchester Royal Infirmary, United Kingdom; **Manchester Academic Health Science Centre, United Kingdom

Introduction: Patients with diabetes have impaired endothelial colony-forming cell (ECFC or endothelial progenitor cell) function leading to poor vascular endothelial repair, potentially contributing to diabetic foot ulceration, a major

health and economic burden. The objective was to characterise the functional deficit in these cells in order to understand the pathogenic mechanism and identify novel therapeutic targets.

Materials and Methods: ECFCs were harvested from the peripheral blood of healthy controls and patients with diabetes and neuroischaemic foot ulcers using cell culture techniques. Functional and biochemical analyses of the migratory, angiogenic and metabolic activity were performed. In addition, a neotissue array was used to compare ECFC binding and growth to different matrix ligands (RGD, DGEA, IKVAV, YIGSR, VAPG) and combinations of ligands, including soft and fibrous matrices to represent distinct environments.

Results: Neuroischaemic ECFCs take longer to form colonies on isolation and have impaired nitric oxide production (46% of control) and a reduced migratory response to SDF-1 (77% of control), decreased scratch closure (87% of control) and compromised tube formation (38% of control in the matrigel assay compared to healthy ECFCs). Seahorse extracellular flux analysis of metabolic function identified no change in glycolysis, but mitochondrial function and maximal oxygen consumption were increased two-fold in the neuroischaemic ECFCs *vs* healthy ECFCs. The neotissue array revealed a decrease in binding of neuroischaemic and control ECFCs to stiff matrix compared to normal physiological stiffness by between a half and two-thirds over 24 h. The neuroischaemic ECFCs bound less, and to a reduced repertoire of matrix peptides compared to controls, suggesting a diminished binding capacity. Furthermore, in long-term culture the viability of neuroischaemic ECFCs was reduced compared to control cells.

Discussion: This study is the first to describe the defect in matrix adhesion and metabolic changes in neuroischaemic diabetic ECFCs, which may contribute to impaired endothelial repair observed *in vivo*. Further work to characterise the mechanism of this binding deficiency will allow us to develop an improved model of disease *in vitro,* leading to identification of new therapeutic targets and stem cell therapies for wound healing.

The *in vitro* effect of syndecan-3 gene knockout on bone marrow-derived mesenchymal stem cells' properties

A. Stefan*; A. G. Kay†; N. Forsyth‡; F. K. Jones§; A. Pisconti§; O. Kehoe*

ISTM, Keele University at RJAH Orthopaedic Hospital, Oswestry SY10 7AG; †Biology Department, University of York, Heslington, York YO10 5DD; ‡The Guy Hilton Research Laboratories, Keele University Medical School, Thornburrow Drive, Hartshill, Stoke on Trent ST4 7QB; §Department of Biochemistry, Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool L69 7ZB

Introduction: Inflammation is a central feature of rheumatoid arthritis that affects around 1% of the population and can result in disability and morbidity. The immunomodulatory effects of bone marrow-derived mesenchymal stem cells (MSCs) have been widely studied, and the recent observations that syndecan-3 (SDC3) is selectively pro-inflammatory in the joint led us to hypothesise that SDC3 might play an important role in MSC biology.

Materials and Methods: MSCs were isolated from bone marrow of C57Bl/6 WT ($n = 6$) and *Sdc3*$^{-/-}$ ($n = 6$) mice and used to assess the following: flow cytometry (immunophenotype, size and complexity analysis); population doubling time; colony-forming units; osteogenic, adipogenic and chondrogenic differentiation; adhesion properties to type II collagen, fibronectin and laminin; and migration properties. Western blotting was used to investigate which signalling pathways are affected by SDC3 loss in mMSCs.

Results: Immunophenotypic analysis indicated similar surface marker expression pattern for both WT and *Sdc3*$^{-/-}$ mMSCs. While both cell types show similar FSC values, the cell complexity in WT mMSCs showed significantly higher values than the *Sdc3*$^{-/-}$. The spread cell surface area of *Sdc3*$^{-/-}$ mMSCs was dramatically lower as compared to WT. The differentiation potential was similar for both WT and *Sdc3*$^{-/-}$ mMSCs. Collagen and fibronectin significantly improved the adhesion of *Sdc3*$^{-/-}$, but not WT, mMSCs. Also, collagen significantly increases the number of *Sdc3*$^{-/-}$ mMSCs when compared with WT cells. Laminin proved to have no effect. The wound healing assay showed no significant difference between the two cell types. More WT MSCs migrated towards serum or pleiotrophin (PTN) in the transwell assay. Interestingly, *Sdc3*$^{-/-}$ mMSCs cultured on collagen showed a dramatic increase in AKT phosphorylation accompanied by a decrease in ERK1/2 phosphorylation compared with WT controls.

Discussion: The significantly reduced complexity expressed by the *Sdc3*$^{-/-}$ mMSCs might be due to the fact that the cytoplasmic domains of syndecans are commonly involved in cytoskeletal regulation; thus, the ablation of *Sdc3*$^{-/-}$ may trigger cellular morphological changes. These morphological changes were confirmed by the significant reduced cell surface spread of *Sdc3*$^{-/-}$ mMSC and appeared to be driven by hyperactivation of the PI3K/AKT pathway at the expense of the ERK1/2 pathway.

Modelling Alport syndrome using patient-derived kidney organoids

P. Tian*; S. J. Kimber*; D. P. Gale†; R. Lennon*

Faculty of Biology, Medicine and Health, University of Manchester, Manchester, United Kingdom; †Centre for Nephrology, University College London, London, United Kingdom

Introduction: Alport syndrome is a rare renal disease caused by mutations in the genes COL4A3, COL4A5 or COL4A5 leading to reduced or absent type IV collagen α3α4α5 networks in glomerular basement membranes (GBM), which underlie all epithelial sheets providing a scaffold for cells.
Although the genetic basis for Alport syndrome has been known for decades, there is incomplete understanding about pathogenic mechanisms. Human pluripotent stem cell (hPSC)-derived kidney organoids have great potential for understanding kidney development and facilitating disease modelling, and ultimately as a source for renal replacement.
Materials and Methods: Induced pluripotent stem cells (iPSCs) derived from patients with Alport syndrome were obtained from the Human Induced Pluripotent Stem Cell Initiative (HipSci), along with healthy control lines. Both Alport iPSCs and control hPSCs were differentiated to kidney organoids following a modified protocol from Takasato et al. in 2015.
Results: Whole-mount immunofluorescence and immunohistofluorescence showed the organoids contain the most of the cell populations in a mature kidney such as podocytes ($WT1^+$, $NPHS1^+$), proximal tubule (LTL^+), distal tubule ($ECAD^+$), and endothelial cells ($CD31^+$), and form an appropriately segmenting nephron structures after 7 days of 2D culture followed by 18 days of 3D culture *in vitro*. Notably, mature GBM proteins laminin and type IV collagen network were expressed surrounding the glomeruli and between nephrons, which were only observed from animal models or *in vivo* transplanted organoids. Real-time PCR data also suggested the present of laminin and type IV collagen networks in GBM by verifying the gene expression level of LAMB1, LAMB2, and COL4A1, COL4A3, COL4A4, COL4A5, and COL4A6. Primitive foot processes were observed by transmission electron microscopy.
Discussion: Although the *in vitro* grown organoids are not fully developed regarding structure and mature function, we have demonstrated that the organoids generated contain the key components of GBM, and therefore would be a promising model for investigation of basement membrane assembly and improvement of mechanistic understanding and would lead to the identification of new therapeutic targets.

A polycaprolactone/glycosaminoglycan scaffold for peripheral nerve regeneration

M. Idini*; P. Wieringa†; G. Nieddu*; A. J. Lepedda*; M. Formato*; L. Moroni†

*Department of Biomedical Sciences, Sassari University, Sassari, Italy; †Department of Complex Tissue Regeneration, Maastricht University, Maastricht, The Netherlands

Introduction: Nerve fibers of the peripheral nervous system (PNS) have a remarkable ability to regenerate and can lead to an almost complete recovery of normal function. This process is governed by glial cells, known as Schwann cells, through their unique capacity to dedifferentiate into cells that drive the healing process. However, post-traumatic nerve repair continues to be a major challenge in restorative medicine and micro-surgery and several studies have been conducted to evaluate the efficiency of acellular nerve grafts. The major challenge in tissue engineering is to develop a synthetic support structure, or scaffold, able to mimic the natural extracellular matrix (ECM). In this respect, it is known that glycosaminoglycans (GAGs) of the extracellular matrix are involved in proliferation, synaptogenesis, neural plasticity and regeneration of the PNS. Here, we developed fibrous scaffolds functionalized with GAGs that allowed us to assess their influence on the adhesion, proliferation, and differentiation of Schwann cells.
Materials and Methods: We set up a method to functionalize electrospun scaffolds of polycaprolactone (PCL) fabricated with both random and aligned fibres with GAGs purified from porcine vascular tissue. Neuronal Schwann cells RT4-D6P2T were seeded on scaffolds with or without GAGs.
Proliferation, metabolic activity, and GAGs assays were performed. The expression of specific markers, that is, Syndecan 1, Syndecan 4, Integrin, Laminin and p75, during 7 days of culture, was evaluated by both immunofluorescence and Western blot analyses.
Results: We found that functionalization with GAGs of both aligned and random fiber scaffolds resulted in increased cell proliferation at day 1. In addition, functionalized aligned scaffolds displayed an increase in GAG levels at day 1, probably due to cell extracellular matrix formation, and an increase in syndecan-4 expression at day 7.
Discussion: Overall, cellular colonization studies suggest that PCL-GAG scaffolds could represent a promising artificial substrate that closely mimics the recently established pattern of Schwann cell migration into the regenerating nerve.

Modelling the brain tumour microenvironment

J. Curd*; J. Ashworth*; A. Grabowska*; B. Coyle†; C. Merry*

*Division of Cancer & Stem Cells, University of Nottingham, Nottingham, United Kingdom; †Children's Brain Tumour Research Centre, School of Medicine, University of Nottingham, Nottingham, United Kingdom

Introduction: Current *in vitro* models used to study children's brain cancer using 2D monolayer cultures or 3D models containing animal-derived products typically fail to recapitulate the complexity of the brain tumour microenvironment, unreliably represent human extracellular matrices (ECM), and/

or raise ethical questions of using animal-derived products in research. Therefore, we aim to optimise a synthetic peptide hydrogel model which can be selectively functionalised with various ECM components to mimic specific tumour microenvironments.

Materials and Methods: The self-assembling FEFEFKFK gelator octapeptide has been used to encapsulate a variety of cell types with excellent viability. Functionalisation of the gel can be achieved by stirring in soluble ECM components during cell encapsulation. We will also apply various additional methods to functionalise the peptide, including direct extension to contain ECM-protein cell-binding motifs and incorporation of non-natural amino acids to enable subsequent addition of functional groups. The effect of these modifications on encapsulated cells will be evaluated using a variety of techniques including fluorescent-confocal microscopy and RT-PCR.

Results: Representative cells have been encapsulated and cultured, including the medulloblastoma cell lines ONS76 and CHLA-01R-MED. We have also established in-gel culture of mouse embryonic stem cell lines E14 and Ext1$^{-/-}$. Differentiation of Ext1$^{-/-}$ cells, lacking endogenous heparan sulphate (HS), has been observed only in gels functionalised with HS, demonstrating the immobilised glycosaminoglycan is biologically active.

Discussion: Initial results demonstrate that relevant encapsulated cell types can be cultured and that functionalisation of the gel is associated with characteristic changes in behaviour of at least one cell type. From this, we have constructed a test environment to evaluate the biological effect of modifications to the hydrogel. Our aim is to develop a model that recapitulates the brain tumour microenvironment in a superior way compared to current models, without the need for animal-derived products, for application in the evaluation of new drug treatments to combat children's brain cancer.

The interaction of thrombospondin-1 and -2 with FGF2 in the control of tumor angiogenesis

P. Borsotti*; D. Pinessi*; L. Carminati*; D. S. Annis†; D. F. Mosher†; G. Colombo‡; M. Rusnati§; G. Taraboletti*

*Tumor Angiogenesis Unit, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Bergamo, Italy; †University of Wisconsin, Madison, WI, USA; ‡ICRM-CNR, Milano and Università di Pavia, Pavia, Italy; §University of Brescia, Brescia, Italy

Introduction: Thrombospondin (TSP)-1 and TSP-2 are major endogenous inhibitors of angiogenesis. They act through different mechanisms, including interaction with endothelial cell receptors and direct binding to angiogenesis regulatory factors. We previously demonstrated that the type III repeat (T3R) domain of TSP-1 inhibits angiogenesis by binding and sequestering the angiogenic factor FGF2. Since TSP-2 shares with TSP-1 similar structures and functional properties, we hypothesized that also TSP-2 might bind FGF2 and that the T3R domain could be used as a model for the development of new inhibitors of pathological angiogenesis.

Materials and Methods: The FGF2 binding properties of TSP-1 and TSP-2, recombinant domains, and synthetic peptides were investigated by solid-phase binding and surface plasmon resonance assays. Computational docking analysis was used to characterize the binding interface. Non-peptidic, TSP-based compounds identified by pharmacophore-based screening of libraries of small molecules were analyzed for ability to bind FGF2 and inhibit its activity in biological assays.

Results: The T3R domain of TSP-2 bound FGF2, with high affinity (*Kd* in the low nanomolar range) and binding properties similar to the T3R domain of TSP-1. Binding was affected by calcium and heparin. The minimal FGF2 interacting sequence was localized in a 7mer peptide of TSP-1 and the corresponding sequence in TSP-2. The interaction of TSP-2 and TSP-1 with FGF2 showed comparable molecular requirements and resulted in the inhibition of FGF2 binding to both heparin (used as a structural analogue of heparan sulfate proteoglycans) and FGFR-1. Small molecules mimetic of TSP-1 prevented the binding of both TSP-1 and TSP-2 to FGF2, confirming the shared recognition determinants, and inhibited FGF2 interaction with endothelial cell receptors and angiogenic activity.

Discussion: This study identifies TSP-2 as a new FGF2 ligand that shares with TSP-1 similar binding properties and molecular requirements and a comparable capacity to block FGF2 interaction with pro-angiogenic receptors. This likely contributes to TSP-2 antiangiogenic and antineoplastic activity, providing the rationale for future therapeutic applications.

Cartilage intermediate layer protein 1 (CILP1): A novel mediator of cardiac extracellular matrix remodelling

F. A. van Nieuwenhoven*; C. Munts*; R. C. Op 't Veld*; A. González†; J. Díez†; S. Heymans‡; B. Schroen‡; M. van Bilsen*

*Department of Physiology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands; †Program of Cardiovascular Diseases, CIMA, University of Navarra, Pamplona, and CIBERCV, Carlos III National Institute of Health, Madrid, Spain; ‡Department of Cardiology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands

Introduction: Heart disease is frequently accompanied by cardiac extracellular matrix (ECM) remodelling, often

leading to cardiac fibrosis. In the present study, we explored the significance of cartilage intermediate layer protein 1 (CILP1) as a novel mediator of cardiac ECM remodelling.

Materials and Methods: Whole-genome transcriptional analyses on human myocardial samples from patients with aortic valve stenosis were performed to determine whether CILP1 expression was associated with cardiac fibrosis. CILP-1 protein levels were determined in human cardiac samples using Western blot. Cardiac CILP1 mRNA levels were assessed by qPCR in mouse models of myocardial infarction and hypertension. The cellular source of myocardial CILP1 expression, its regulation and effect were studied in isolated human and rat cardiac cells.

Results: Transcriptional analysis of human cardiac tissue samples revealed a strong association of CILP1 with many structural (eg, COL1A2 $r^2 = 0.83$) and non-structural (eg, TGFB3 $r^2 = 0.75$) ECM proteins. Gene enrichment analysis further underscored the involvement of CILP1 in human cardiac ECM remodelling and TGFβ signalling. Myocardial CILP-1 protein levels were significantly elevated in human infarct tissue and in aortic valve stenosis patients. CILP1 mRNA levels markedly increased in mouse heart after myocardial infarction, transverse aortic constriction, and angiotensin II treatment. Cardiac fibroblasts were found to be the primary source of cardiac CILP1 expression. Recombinant CILP1 inhibited TGFβ-induced αSMA gene and protein expression in cardiac fibroblasts. In addition, CILP1 overexpression in HEK293 cells strongly (5-fold $P < 0.05$) inhibited TGFβ signalling activity.

Discussion: In conclusion, our study identifies CILP1 as a new cardiac matricellular protein interfering with pro-fibrotic TGFβ signaling, and as a novel sensitive marker for cardiac fibrosis.

High-affinity collagen binding and signalling requires clustering of DDR1 in the cell membrane

D. Corcoran; V. Juskaite; B. Leitinger

National Heart and Lung institute, Imperial College London, United Kingdom

Introduction: The collagen receptor discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase with key functions in cell regulation, including adhesion, migration and proliferation. DDR1 is considered an attractive therapeutic target for a number of human diseases, but little is understood about how collagen binding results in intracellular kinase activation. In contrast to classical receptor tyrosine kinases, whose kinase activity is switched on within seconds of ligand binding, DDR1 activation is slow, with autophosphorylation occurring within hours of collagen binding. Our previous studies defined the interaction of the extracellular discoidin domain with the collagen triple helix at atomic-level detail, but how this translates to collagen binding to DDR1 on the cell surface has not been explored.

Materials and Methods: DDR1 distribution and collagen binding on the cell surface were visualised with immunofluorescence. Flow cytometry was used to measure collagen and collagen-mimetic peptide binding to DDR1. DDR1 autophosphorylation was visualised with Western blotting.

Results: Here, we show that collagen binding results in clustering of DDR1 in the cell surface with kinetics that are much faster than kinase activation. DDR1 clustering is induced by stimulation with different collagen types as well as collagen-mimetic peptides. DDR1 mutations that block signalling but are far away from the ligand-binding pocket block clustering and collagen binding but do not affect binding of collagen-mimetic peptides. Blocking antibodies that modulate signalling allosterically inhibit collagen-induced clustering and collagen binding to cells.

Additionally, we show that at longer stimulation times, clustered DDR1 redistributes into a more aggregated state which correlates with autophosphorylation.

Discussion: We conclude that high-affinity collagen binding requires DDR1 clustering in the cell membrane, which is dependent on discoidin domain residues far away from the collagen-binding pocket, as well as intact transmembrane helix interactions. Thus, receptor-receptor interactions are required for high-affinity collagen binding to DDR1. Autophosphorylation results from a second-stage, slow DDR1 redistribution into a more aggregated state, indicating kinase activity requires high molecular density.

Insights into the structure and dynamics of lysyl oxidase propeptide, a flexible protein with numerous partners

S. D. Vallet*; A. E. Miele*; U. Uciechowska-Kaczmarzyk†; A. Liwo†; B. Duclos*; S. A. Samsonov†; S. Ricard-Blum*

**University Lyon 1, CNRS, Institute of Molecular and Supramolecular Chemistry and Biochemistry, UMR 5246, Villeurbanne cedex, France; †Laboratory of Molecular Modeling, Department of Theoretical Chemistry, Faculty of Chemistry, University of Gdansk, Poland*

Introduction: Lysyl oxidase (LOX) catalyzes the oxidative deamination of lysine and hydroxylysine residues in collagens and elastin. It is secreted as a proenzyme, which is cleaved by bone morphogenetic protein-1 into the LOX catalytic domain and the N-terminal propeptide (LOX-PP). LOX-PP has anti-tumoral and pro-adipogenic activities.

Materials and Methods: The recombinant human LOX-PP was characterized by circular dichroism (CD),

dynamic light scattering (DLS), and small-angle X-ray scattering (SAXS). Five models of the propeptide were built by coarse-grained molecular dynamics simulations restrained by SAXS data. Binding partners of LOX-PP were identified by surface plasmon resonance and biolayer interferometry.

Results: LOX-PP is an elongated protein (Dmax: 11.7 nm) fitting to an envelope of 14.3 nm × 6.3 nm × 5.2 nm. The ratio of its radius of gyration, determined by SAXS (3.7 nm), to its hydrodynamic radius, calculated by DLS (3 nm), is higher than 0.8, which indicates that LOX-PP is extended. This is consistent with the presence of intrinsic disorder (64.5% evaluated by CD) and the existence of several conformations based on Ensemble modelling. The N-terminus of LOX-PP appears to be folded in 4 out of the 5 models. LOX-PP is indeed able to fold into a-helix in the presence of trifluoroethanol, and the binding of a hexasaccharide of heparin is predicted to induce partial folding of the propeptide. Heparin-binding sites appeared to be mostly localized at the N-terminus. We have identified 19 new binding partners of the propeptide, including fibrillar collagens, glycosaminoglycans, cross-linking and proteolytic enzymes, one proteoglycan, one growth factor and one membrane protein.

Discussion: Our data suggest new roles for the propeptide in ECM assembly and cross-linking, cell-matrix adhesion, and in the regulation of signaling pathways.

Recycled secreted metalloproteases are required for formation of the primary cilium and hedgehog signalling

S. Nandadasa*; L. W. Wang*; A. O'Donnell*; C. M. Kraft*; R. Patel*; H. Y. Gee†; K. Grobe‡; T. C. Cox§; F. Hildebrandt¶; S. S. Apte*

*Cleveland Clinic Lerner Research Institute, Cleveland, OH, USA; †Yonsei University College of Medicine, Seoul, South Korea; ‡University of Münster, Münster, Germany; §University of Washington, Seattle, USA; ¶Harvard Medical School, Boston, MA, USA

Introduction: The primary cilium, an organelle present in most cells, has an indispensable role in cell signalling. Here, the secreted, homologous metalloproteases ADAMTS9 and ADAMTS20 are identified as novel regulators of ciliogenesis and developmental hedgehog signalling.

Materials and Methods: The study utilized combinatorial mouse ADAMTS9 alleles and combined mutagenesis of ADAMTS9 and ADAMTS20, RNA in situ hybridization, immunofluorescence of extracellular matrix and cellular organelles, super-resolution microscopy, scanning and transmission electron microscopy, yeast-two-hybrid cloning, CRISPR-Cas9 inactivation of ADAMTS9 in RPE-1 cells, LRP and clathrin siRNA and rescue by recombinant ADAMTS proteases.

Results: Combined ADAMTS9 and ADAMTS20 mutagenesis in mice led to an open neural tube with defective hedgehog signalling, which is transduced by primary cilia. In addition to extracellular matrix (CSPG, HSPG and fibronectin) accumulation in the neural tube, consistent with the cognate proteolytic activity of ADAMTS9 and ADAMTS20 against proteoglycans, mutant neural epithelial cells had short primary cilia.

Super-resolution microscopy of several cell types identified ADAMTS9 and ADAMTS20 in $Rab11^+$ vesicles encircling the base of cilium. Trafficking analysis showed that vesicular ADAMTS9 was derived from secreted and furin-processed ADAMTS9 initially bound to the cell surface and subsequently internalized by LRP1 and clathrin-mediated endocytosis. Ciliogenesis was impaired by CRISPR-Cas9 inactivation of *ADAMTS9* in RPE-1 cells and restored by either transfected or exogenous ADAMTS9 and ADAMTS20, which trafficked to the cilium base. Catalytically active ADAMTS9 or ADAMTS20 but not their proteolytically inactive mutants nor related matrix-degrading proteases ADAMTS1 and ADAMTS5 could rescue the defect in mutant RPE-1 cells. Imaging of sequential steps in ciliogenesis using super-resolution microscopy and electron microscopy suggested that endocytosed ADAMTS9 and ADAMTS20 mediate ciliary vesicle expansion and uncapping of the mother centrosome, which are prerequisites for ciliary axoneme extension. Analysis of pericellular proteoglycans in RPE-1 cells suggested an uncoupling of ECM proteolysis from ciliogenesis.

Discussion: The findings show that in addition to a canonical role in matrix proteolysis, ADAMTS9 and ADAMTS20 have an unexpected and independent role in formation of the primary cilium. Their dual function is ensured by initial secretion and cell-surface binding for pericellular ECM proteolysis and subsequent internalization to a unique pericentriolar vesicle population mediating ciliogenesis.

Unusual collagens in the *C. elegans* matrisome—a new classification of cuticular collagens

A. Teuscher*; A. Naba†; C. Y. Ewald*; J. M. Gebauer‡

*Eidgenössische Technische Hochschule (ETH) Zürich, Health Sciences and Technology, Schwerzenbach-Zürich, Switzerland; †Department of Physiology and Biophysics, University of Illinois at Chicago, College of Medicine, Chicago, IL 60612, USA; ‡Institute of Biochemistry, University of Cologne, Cologne, Germany

Introduction: Collagens form the most abundant protein family in the human body and are important in development, homeostasis and disease. Recently, we demonstrated that they

are also important for longevity in *C. elegans* (Ewald et al. Nature 2015). In analogy to the efforts to characterise all extracellular matrix (ECM) proteins as well as ECM-associated proteins in humans (termed "matrisome" (Naba et al. MCP 2012)), we used bioinformatic methods to define the matrisome in *C. elegans*. Here, we focus on the characterisation of collagens and their unusual organisation in nematodes.

Materials and Methods: We identified collagens by a combination of bioinformatics analysis and manual curation. Classification was performed by manual pattern recognition assisted by bioinformatics.

Results: With 184 members, the category of collagens is the largest category in the core matrisome of *C. elegans*. Interestingly, true homologues are only present for collagen IV. Six other collagens have similarities in terms of their domain arrangements but do not share any significant sequence similarity. Strikingly, classical fibrillar collagens (like collagen I or II) are completely absent in *C. elegans*.

A total of 172 collagens belong to the group of cuticular collagens, defined by a relatively short collagenous domain (approx. 40 GXY triplets), which is flanked by an N- and C-terminal cysteine knot. Similar to fibrillar collagens, there is an additional N-ProHelix of usually 10 GXY repeats stabilised by another cysteine knot. We further clustered these collagens into 4 main groups, subdivided into 74 clusters—based on the idea that protein within one cluster should be more likely to form heterotrimers. Many (117) cuticular collagens are predicted to be transmembrane; however, most of them carry a furin cleavage site, indicating shedding. Interestingly, there is no obvious C-Propeptide suggesting other mechanisms of chain selectivity and probably even triple helix formation.

Discussion: Our proposed classification helps understand observed phenotypes as well as identifying promising candidate genes for future investigations. We already started initial experiments to determine the spatio-temporal localisation of collagens by GFP-fusion constructs, as well as analysis of the biochemical characteristics of these unusual collagens. Together with the newly defined matrisome, this classification will help interpret future findings in genetic screens and proteomic analyses.

Differential dynamics of fibrillar collagen-binding integrins in cell-collagen interactions

M. Moses*; P. Erusappan[†,‡]; C. Zeltz[§]; C. McCulloch[¶]; D. Gullberg*

*Department of Biomedicine, University of Bergen, Bergen, Norway; [†]Institute for Experimental Medical Research, Oslo University Hospital and University of Oslo, Oslo, Norway; [‡]KG Jebsen Cardiac Research Center and Center for Heart Failure Research, University of Oslo, Oslo, Norway; [§]Princess Margaret Cancer Center, University Health Network, Toronto, Ontario, Canada; [¶]Matrix Dynamics Group, University of Toronto, Toronto, Ontario, Canada

Introduction: Integrins α2β1 and α11β1 are two collagen receptors which in addition to sharing sequence similarity also share similar preference for fibrillar collagens, suggesting that these integrins could have similar functions when co-expressed in mesenchymal cells. Available data however suggest that these collagen-binding integrins have different functions both *in vitro* and *in vivo*. Although both integrins are expressed on fibroblasts, only α11β1 has been reported to contribute to myofibroblast differentiation and to regulate cardiac and wound fibrosis. How these two integrins transduce distinct functions is unclear.

Material and Methods: We investigated the potential differences between the two fibrillar collagen-binding integrins, α2β1 and α11β1, with a special focus to their distribution in cell-matrix adhesions using primary human gingival fibroblasts and C2C12 cells expressing chimeric constructs, where the cytoplasmic tail of integrin α2 was swapped with α11 cytoplasmic tail (α2Xα11C-mcherry) or vice versa (α11Xα2C-EGFP).

Results: Integrins α2β1 and α11β1 displayed different cell surface dynamics during matrix remodeling and assembly in human gingival fibroblasts. Whereas α2β1 integrin in spreading fibroblasts remained localized to focal adhesions at the periphery of the cells, α11β1 appeared to slide from focal adhesions into central adhesions. Integrin α11β1 in central adhesions co-localized both with its ligand, fibrillar collagen type I, and the fibrillar adhesion markers tensin-1 and Kank2, thus establishing α11β1 integrin as a novel marker for fibrillar adhesions. To better understand the molecular mechanism explaining why α11β1, but not α2β1, translocates into fibrillar adhesions, we exchanged the cytoplasmic tail of integrin α2 with that of α11, and vice versa. Replacing integrin α11 cytoplasmic tail with the cytoplasmic tail of α2 (α11Xα2C-EGFP) strongly reduced α11β1 fibrillar adhesions, whereas replacing α2 cytoplasmic tail with the α11 cytoplasmic tail (α2Xα11C-mcherry) did not result in integrin α2β1 becoming localized in fibrillar adhesions.

Discussion: Our results strongly suggest that both the extracellular part and cytoplasmic tail of integrin α11 are involved in the α11β1-dependent stabilization of fibrillar adhesions, and further studies are needed to identify the molecular mechanisms underlying the differential distribution of α2β1 and α11β1.

Integrin α11 cytoplasmic tail is required for FAK activation to initiate 3D cell invasion and ERK-mediated cell proliferation

P. Erusappan*[,†]; C. Zeltz[‡]; N. Lu[§]; D. Gullberg[§]

*Institute for Experimental Medical Research, Oslo University Hospital and University of Oslo, Oslo, Norway; [†]KG Jebsen Cardiac Research Center and Center for Heart Failure Research, University of Oslo, Oslo, Norway; [‡]Princess Margaret Cancer Center, University Health Network, Toronto, Ontario, Canada; [§]Department of Biomedicine, University of Bergen, Bergen, Norway

Introduction: Integrin α11β1 is a collagen-binding integrin, which is receiving increasing attention in the context of wound healing, fibrosis and tumour-stroma interactions. Integrin α11β1 has been reported to contribute to myofibroblast differentiation and could be a potential target to control cardiac fibrosis. The detailed molecular mechanisms through which integrin α11β1 mediates fibroblast cell functions are poorly understood. To better understand the signaling function of integrin α11β1, we addressed this question by characterizing the role of integrin α11 cytoplasmic tail.
Materials and Methods: We investigated the role of integrin α11 cytoplasmic tail by generating a truncated variant of α11 with a deletion of the terminal 17 amino acid residues in the α11-tail. C2C12 cells lacking collagen-binding integrins were transfected with wild-type α11 or α11-tail-less constructs to study the effect of this deletion in integrin α11 function.
Results: C2C12 cells expressing tail-less α11 attached normally to collagen I, but displayed fewer focal contacts on collagen I. Integrin α11-tail-less cells furthermore displayed a reduced capacity to invade and reorganize a 3D collagen gel and proliferated less when compared to wild-type cells. Analysis of cell signaling showed that α11-mediated FAK and ERK phosphorylation was reduced in cells expressing tail-less α11. ERK and FAK inhibitors inhibited α11-mediated cell proliferation, whereas α11-mediated cell invasion was FAK-dependent and occurred independently of ERK signaling.
Discussion: Our data demonstrate that the integrin α11 cytoplasmic tail plays a central role in α11 integrin specific functions, including the FAK-dependent ERK activation, and suggest that α11-tail can regulate fibroblast adhesion, migration, and proliferation in a fibrillar collagen matrix.

Chondrocyte specific deletion of CCN2 does not exacerbate osteoarthritis in models of post-traumatic osteoarthritis in mice

C. Keenan*; M. Jaffa*; P. Milner*; I. Kanakis*; A. Carter*; D. Abraham†; A. Leask‡; R. Van't Hof*; G. Bou-Gharios*; B. Poulet*

*Department of Musculoskeletal Biology I, William Henry Duncan Building, Institute of Ageing and Chronic Disease, University of Liverpool, United Kingdom; †Centre for Rheumatology and Connective Tissue Diseases, University College London, United Kingdom; ‡Schulich School of Medicine and Dentistry, Western University, Ontario, Canada

Introduction: CCN2 is a matricellular protein expressed in both healthy and osteoarthritic cartilage. It enhances the production of aggrecan and collagen type II, whilst also promoting the proliferation, differentiation, and maturation of growth-plate chondrocytes. CCN2 null mice exhibit a range of skeletal dysmorphisms, highlighting the importance of CCN2 in regulating matrix formation and turnover. The aim of this study was to determine the function of CCN2 in chondrocytes in models of trauma-induced osteoarthritis (OA).
Materials and Methods: CCN2 gene deletion was induced specifically in chondrocytes following tamoxifen treatment in male CCN2 floxed mice aged 8 weeks. OA was induced either through surgical injury, or non-invasively by applying a controlled loading programme, to the tibio-femoral joint. Knee joints were harvested, scanned with μCT, and processed for histology. Sections were stained Safranin O or toluidine blue and scored for cartilage degradation using the OARSI grading system.
Results: In the surgical model of OA, cartilage degeneration was more severe in the medial tibia of CCN2 KO mice compared to WT with maximum scores averaging 5.28 (±0.28 SEM) and 4 (±0; $P = 0.02$) for KO and WT respectively, at 4 weeks post-surgery. No significant differences were observed at 8 weeks post-surgery. μCT analysis showed no significant differences in subchondral bone thickness, trabecular bone BV/TV, and trabecular thickness between WT and KO mice at either time point. In the non-surgical model of OA, no significant differences in the severity of OA lesions were observed between KO and WT at 6 weeks post-loading. μCT analysis showed no significant differences between KO and WT loaded knees.
Discussion: The preliminary data obtained suggest that CCN2 may play a protective role in limiting cartilage degeneration in the early stages following initiation of OA; however, this effect appears to be lost with time. The progression of OA in both models at later time points suggests that CCN2 expression in chondrocytes does not prevent OA; however, analysis of earlier time points may reveal further beneficial effects of CCN2 and its role in OA pathogenesis.

Identifying the binding sites of the small ECM protein dermatopontin on fibrillary collagens

M. M. Jensen*; A. Bonna†; S. Hamaia†; R. Farndale†; H. Karring*

*University of Southern Denmark, Department of Chemical Engineering, Biotechnology and Environmental Technology, Odense, Denmark; †Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom

Introduction: Dermatopontin (DPT) is a 22-kDa acidic collagen-binding protein that enhances collagen fibrillogenesis *in vitro* and is retained on the fibrils after fibrillogenesis. However, the binding sites on collagens are unknown. DPT also interacts with a large range of other ECM proteins, growth factors and cell surface receptors. DPT has been hypothesized to act as an adaptor between cells and ECM due to

the interactions with ECM proteins, both fibrous and smaller, and cell surface receptors. DPT plays a role in wound healing and is involved in several fibrotic disorders. Previous studies have shown that knockout of *Dpt* in mice results in thinner dermis and cornea, abnormal fibrils, changes in fibril organization and an Ehlers-Danlos syndrome-like phenotype.

Materials and Methods: DPT was extracted from six different collagenous tissues and purified from the cornea. DPT's binding sites on fibrillary collagen are revealed through the use of the Collagen Toolkits. The Collagen Toolkits are collections of overlapping triple-helical peptides covering the triple-helical domains of collagen II and III. The peptides were arrayed in 96-well plates, together with DPT, in order to map the binding sites.

Results: Extractions from collagenous tissues suggest that the highest amount of DPT is found in the cornea, which was consequently chosen as the source for obtaining purified endogenous DPT for further characterization of the protein. Far-Western blotting of tissue extracts, utilizing DPT as the probe protein, reveals an affinity for different types of collagens. Results from using the Collagen Toolkits reveal several binding sites along the triple-helical domains varying in affinity. Further experiments and data treatment indicate that specificity for these sites is based on a combination of ionic and hydrophobic interactions.

Discussion: The discovered binding sites may contribute to understand DPT's functions *in vivo* and suggest an interplay with other collagen-binding proteins. The identified binding sites could also provide an explanation for why *Dpt* knockout introduces an Ehlers-Danlos syndrome-like phenotype in mice and consequently a possible role of DPT in this disorder in humans.

Distribution and production logistics for *de novo* basement membrane formation

Y. Matsubayashi*; A. Louani*; A. Dragu*; B. Sánchez-Sánchez*; E. Serna-Morales*; L. Yolland*; A. Gyoergy†; G. Vizcay‡; R. Fleck‡; J. M. Heddleston§; T.-L. Chew§; D. Siekhaus†; B. Stramer*

Randall Centre for Cell and Molecular Biophysics, King's College London, London, SE5 9AP United Kingdom; †Institute of Science and Technology, Am Campus 1, 3400 Klosterneuburg, Austria ; ‡Centre for Ultrastructure Imaging, King's College London, London, SE5 9AP, United Kingdom; §Advanced Imaging Center, Howard Hughes Medical Institute, Janelia Research Campus, Ashburn VA 20147, USA

Introduction: The basement membrane (BM) is a thin layer of extracellular matrix (ECM) beneath nearly all epithelial cell types that is critical for cellular and tissue function. It is composed of numerous components conserved among all bilaterians; however, it is unknown how a fully mature BM is constructed in the living animal, with all the components prepared at the appropriate amount, time, and place.

Materials and Methods: To address these questions, here we exploit our ability to live image and genetically dissect *de novo* BM formation during *Drosophila* development, which allows us to examine production and distribution of core BM components from their initial induction in the embryo. Taking into account the precise production dynamics of core BM components, we also mathematically model their synthesis and degradation rates allowing us to predict BM turnover.

Results: We show that migrating macrophages (hemocytes) produce and deliver the majority of BM components throughout the embryo. Failure in this delivery leads to an uneven distribution of ECM, morphogenetic defects, and embryonic lethality. Furthermore, we reveal that hemocytes regulate their temporal expression of specific BM components to allow for their proper incorporation. Finally, mathematical modelling predicts that rapid turnover (half-life = ~17 h) is critical to define the expression levels of BM components. Indeed, manipulating matrix metalloprotease (MMP) expression in the developing embryo alters collagen IV expression dynamics as predicted by the model and affects morphogenesis of the developing nerve cord, which is dependent on BM.

Discussion: Taken together, these results reveal that *de novo* BM construction *in vivo* requires a combination of both production and distribution logistics allowing for the timely supply and incorporation of core components. Moreover, the rapid turnover of the BM reveals that BM is a far more dynamic structure than previously thought.

The role of the circadian clock in the homeostasis of the extracellular matrix in cartilage and intervertebral discs

M. Dudek*,†; N. Yang*,†; J. P. D. Ruckshanthi*,†; N. Gossan*; H.-J. Im‡; Y. Fukada§; K. Yagita¶; J. F. Bateman**; R. P. Boot-Handford†; J. A. Hoyland††,‡‡; Q.-J. Meng*,†

*Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom; †Wellcome Trust Centre for Cell Matrix Research, University of Manchester, Manchester, United Kingdom; ‡Department of Biochemistry, Rush University Medical Center, Jesse Brown VA Medical Center, Chicago, Illinois, USA; §Department of Biological Sciences, The University of Tokyo, Tokyo, Japan. ; ¶Department of Physiology and Systems Bioscience, Kyoto Prefectural University of Medicine, Kyoto, Japan; **Murdoch Children's Research Institute, Parkville, Victoria, Australia; ††Faculty of Medical and Human Sciences, University of Manchester, Manchester, United Kingdom; ‡‡NIHR Manchester Musculoskeletal Biomedical Research Unit, Manchester Academic Health Science Centre, Manchester, United Kingdom*

Introduction: The circadian clock is a molecular mechanism that allows organisms to anticipate the daily changes in their activity driven by the night and day cycle. The rest/activity pattern of behaviour is reflected in tissues on the level of

gene transcription, translation and protein degradation. We hypothesised that the circadian clock would be of particular importance in rhythmically loaded tissues, such as the cartilage and intervertebral discs (IVDs) that need to maintain their vast extracellular matrix (ECM) after bouts of daily activity.

Materials and Methods: We utilised real-time bioluminescence recording of PER2::Luc clock reporter mouse tissue explants to monitor the dynamics of the circadian clock in cartilage and IVDs. RNA sequencing and mass spectrometry was used to reveal the rhythmic transcriptome and proteome. *Col2a1* specific Bmal1 KO ("clockless") mouse model was used to investigate the consequences of disruption of the circadian rhythm.

Results: We show the first evidence that cartilage and IVDs possess circadian rhythm and that it is affected by aging and disrupted by pro-inflammatory cytokines. Our unpublished results show that it is also responsive to daily changes in osmolarity of the ECM and to mechanical loading. Time series RNAseq of mouse cartilage and IVDs revealed hundreds of tissue-specific rhythmic genes. Circadian disruption by deletion of Bmal1 in Col2a1-expressing cells resulted in loss of rhythmic gene expression and dysregulation of key metabolic pathways. Time series mass spectrometry of WT mouse hip cartilage shows for the first time that cartilage proteins, including dozens of ECM proteins, exhibit a diurnal pattern of abundance. Histological analysis of the Bmal1 KO mouse phenotype revealed age-dependent degeneration of the knee cartilage and fibrosis and calcification of IVDs.

Discussion: Taken together, our data suggest that the circadian rhythm is an essential modulatory mechanism for cartilage and IVD physiology, regulating gene expression and protein abundance on a daily basis. Moreover, osmotic and mechanical signalling is involved in setting the pace of the musculoskeletal circadian clock. Coupling of metabolism with the activity pattern allows these tissues to efficiently synthesise or remodel their ECM at the right time and maintain tissue homeostasis.

Monitoring collagen fibre formation and turnover using CRISPR/Cas9 knock-in of Dendra2

A. Pickard; A. Adamson; Y. Lu; J. Chang; R. Garva; N. Hodson; K. E. Kadler

Wellcome Trust Centre for Cell-Matrix Research, University of Manchester

Introduction: Collagen accounts for 30% of body mass where it functions as a major tissue scaffolding protein and stress-shields cells from destructive mechanical forces. Dysregulation in the amount of collagen is associated with fibrosis (excess collagen) and tissue degenerative (low collagen), for which few therapies and treatments are currently available. Understanding how collagen is produced, processed and regulated is key to developing new treatment strategies. A major technical hurdle has been the absence of a robust method to quantitatively image, in living cells, the production of newly formed collagen, and no high-throughput assay exists.

Materials and Methods: Here, we have generated mouse 3T3 cells that have been modified using CRISPR/Cas9 to integrate a Dendra2 fluorescent fusion protein downstream of the signal peptide of the COL1A2 protein. Integration of the reporter maintains the natural context of the genomic structure, ensuring gene expression patterns are maintained. Dendra2 is photoswitchable, which naturally fluoresces green, until exposed to 405-nm light which converts the reporter a red fluorescent protein. With this approach, we can selectively remove the background collagen signal and then image or assay specifically for the production of new "green" collagen.

Results: A first result showed that a bulk of newly formed procollagen traffics through the Golgi apparatus in ~3 h, and this flux occurs once during 24 h. This approach allows measurement of the dynamics of procollagen translation and vesicle secretion whilst under endogenous control mechanisms. Dendra2-collagen fibres form following 4 days in culture in the presence of L-ascorbic acid. Fibres are formed within the cell boundary, and cells then migrate away from the formed fibrils. Furthermore, individual cells are able to produce multiple fibres. The vast majority (> 95%) of collagen fibrils remain stable for up 7 days; however, some fibrils are broken down by cells over the course of 24 h.

Discussion: In conclusion, CRISPR/Cas9 technology has enabled us, for the first time, to live image fibroblasts synthesising collagen fibres at the cell-matrix interface. This technology is likely to be applicable to the study of other fibril-forming collagens or matrix macromolecules.

Honeycomb-like convex structures at the bottom of the dermal layer function as "anchoring structure" to maintain skin elasticity and morphology

T. Ezure*; S. Amano*; K. Matsuzaki†

**Shiseido Global Innovation Center, Kanagawa, Japan; †Department of Plastic and Reconstructive Surgery, International University of Health and Welfare, School of Medicine, Narita, Japan*

Introduction: The dermal layer is composed of abundant extracellular matrix and contributes to skin firmness and morphology. These functions require that the dermal layer

should be properly anchored to subcutaneous tissue, but little is known about how this is achieved. Here, we aimed to clarify the mechanism involved.

Materials and Methods: Ninety facial and 70 body (upper arm and abdominal) skin specimens were prepared from surplus skin excised during plastic surgery. Dermal layer structures were observed three-dimensionally (3D) by micro-X-ray computed tomography (micro-CT), and their composition was identified immunohistochemically. Skin-retaining force was measured non-invasively with a Cutometer MPA 580® (6 mm probe), and movement of the internal skin structures was observed by ultrasonography. We also examined their relationship to skin morphology, evaluated in terms of sagging (ptosis) severity at the cheek of female volunteers.

Results: Micro-CT observation of the 3D structure of the skin specimens revealed that facial skin contains characteristic convex structures at the bottom of the dermal layer. They were connected to form an overall honeycomb-like appearance. Scanning electron micrography (SEM) showed that the convex structures contain vertically directed collagen fibers to the skin surface, in contrast to the bulk dermal layer, where the collagen fibers are directed horizontally. EVG staining revealed similar orientations of elastic fibers. Thus, the convex structures may serve to retain the dermal layer vertically. Indeed, when we pulled up the skin vertically, we found that the dermal layer is retained tightly on subcutaneous tissues through the convex structures. The retaining force, measured with a cutometer (-Uv/Ue), was significantly positively related to the depth of the convex structures in the cheek of female volunteers. Furthermore, facial sagging severity was significantly negatively related to the depth of these structures. The convex structures significantly decreased with aging, and the decrease was significantly related to the degree of facial sagging.

Discussion: Honeycomb-like convex structures appear to serve as "anchoring structure" to retain the dermal layer, contributing to skin elasticity and superficial morphology. They could be a key to understanding the function of the dermal layer.

In situ repair of full-thickness cartilage defects using biomimetic biofunctionalised implants

D. Buttle*; J. Haycock†; M. Pead‡; R. Meeson‡,§; G. Blunn§; A. Crawford¶

**Department of Infection, Immunity & Cardiovascular Disease, University of Sheffield; †Department of Materials Science & Engineering, University of Sheffield; ‡Department of Clinical Sciences and Services, Royal Veterinary College; §Institute of Orthopaedics and Musculoskeletal Science, University College London; ¶School of Clinical Dentistry, University of Sheffield*

Introduction: Cells use the heparin interactome as a "toolkit" for phenotypic change. We hypothesised that taking a biomimetic approach may aid healing of traumatic and early osteoarthritic cartilage.

Materials and Methods: Poly-L-lactic acid random-fibre scaffolds were electrospun and the surface charge was altered by allyl amine plasma polymerisation followed by charge adsorption of heparin to which pmol amounts of TBFβ3 and CXCL12 were non-covalently bound. The scaffold was assessed *in vitro* for support of long-term cell viability and chondrogenesis by bone marrow mesenchymal stem cells (MSCs) and primary chondrocytes. Cartilage formation was determined by measuring glycosaminoglycan content. The scaffolds were assessed for *in vivo* efficacy by implantation into surgically created full-thickness chondral lesions in the medial femoral condyles of sheep. Subchondral bone micro-fracture was used to release bone marrow MSCs into the joint. At 4 and 16 weeks, implanted joints were retrieved and cartilage regeneration assessed macroscopically and histologically.

Results: *In vitro*, functionalisation of the PLLA scaffold with a combination of TGFβ3 and CXCL12 promoted MSC attachment and ingress throughout the implant, and chondrogenic differentiation. Viability of the cells within the construct was maintained for at least 5-6 weeks in the absence of added serum/growth factors. The MSCs underwent chondrogenesis and produced significantly more ECM than non-functionalised or partially functionalised scaffolds. *In vivo*, implantation of TGFβ3- and CXCL12-functionalised implants, but not empty defects or control, non-functionalised implants, showed regeneration of hyaline neocartilage occurring as early as 4 weeks.

Discussion: These results demonstrate the potential utility of heparin interactome proteins in the regeneration of diseased or injured tissues. It is feasible to develop off-the-shelf medical devices for the repair of injured or early osteoarthritic cartilage thereby bypassing potential problems associated with cell therapies.

The effect of [-1A]TIMP3 overexpression on bone mass in the naturally developing OA STR/Ort mice

I. Kanakis; R. van't Hof; K. Liu; A. Pitsillides; G. Bou-Gharios

Institute of Ageing and Chronic Disease, Faculty of Health & Life Sciences, University of Liverpool, William Duncan (Apex) Building, 6 West Derby Street, L7 8TX, Liverpool, United Kingdom

Introduction: Osteoarthritis (OA) is the most prevalent degenerative joint disorder that affects a large proportion of ageing population, for which there is no definitive treatment. OA progression affects both articular cartilage and bone integrity, including osteophyte formation and subchondral bone thickness.

It has been previously shown that [-1A]TIMP3 (TIMP-3 with an extra alanine residue at -1 residue) can selectively inhibit ADAMTS-4 and -5, while it is a poor inhibitor of collagenases. We sought to investigate the *in vivo* effect of [-1A]TIMP3 overexpression on bone tissue in a spontaneously developing OA mouse model (STR/Ort mouse) and evaluate the impact of long-term aggrecanase inhibition on bone in OA treatment.

Materials and Methods: We generated several transgenic mouse lines that stably overexpressed [-1A]TIMP3, either conditionally in chondrocytes (using col2a1 promoter) or in all tissues (ELF1-promoter) using lentiviral constructs, in the STR/Ort mouse background. Mice were screened for the [-1A]TIMP3 overexpression by qPCR and bred to establish different levels of TIMP-3. At 40 weeks of age, mice were sacrificed and hindlimbs scanned by μCT for bone microarchitecture evaluation and decalcified and processed for histological analyses of the knee joints. Adult bone cells were also infected with [-1A]TIMP3 to validate the effect.

Results: 34 STR/Ort mice were examined by μCT measuring the tibial trabecular bone parameters of the distal metaphysis and compared with wild-type age matched STR/Ort and mice with B6CBA mixed background. [-1A]TIMP3 mRNA expression in the hips did not differ in the STR/Ort mice in terms of gender and site (left/right). Bone mass was substantially higher in STR/Ort mice as compared with WT, and in STR/Ort females in comparison with males, in both conditional and global overexpression. *In vitro* data show significant increases in mineralization of osteoblasts infected with [-1A]TIMP3 compared with TIMP3 and empty virus.

Discussion: We have previously shown that TIMP-3 overexpression leads to loss of bone mass. Data presented here show that [-1A]TIMP3, which inhibits primarily aggrecanases, leads to increased bone mass. This suggests that the balance between aggrecanases and collagenases during bone formation or even adult bone is critical to maintaining healthy bone. It also suggests that [-1A]TIMP3 may potentially be useful in osteoporosis.

The role of the circadian clock-regulated vacuolar protein sorting (VPS) tethering complex in collagen i turnover and cell contractility

J. Chang; R. Garva; A. Pickard; K. E. Kadler

Wellcome Trust Centre for Cell-Matrix Research, Division of Cell Matrix Biology and Regenerative Medicine, Faculty of Biology Medicine and Health, The University of Manchester, Manchester, M13 9PT, United Kingdom

Introduction: Deregulation of extracellular matrix (ECM) forms the basis to many pathological conditions, including fibrosis, tendinopathies, and cancer. Fibroblasts are the major cell type that produces ECM in the body; as such, understanding how ECM production and turnover is controlled in fibroblasts is key to discovering processes that could be targeted for potential therapeutics. Previously we have identified that ECM production and turnover in fibroblasts is regulated by the circadian rhythm. In particular, a member of the vacuolar protein sorting (VPS) family of tethering complexes in protein sorting, VPS33b, is found to be rhythmic from RNA microarray data. VPS33b has been implicated in post-Golgi sorting of protein cargoes, as well as maturation of endosomes/lysosomes. Here, we hypothesise that VPS33b is crucial to the circadian control of both collagen I secretion and uptake.

Materials and Methods: Using CRISPR-Cas9 and shRNA, we have knocked out VPS33b expression in immortalised Per2-luc mouse tail tendon fibroblasts. Immunofluorescence staining, collagen contraction assay, scratch-wound assay, and LumiCycle analyses were carried out. Proteomics studies were also carried out on tail tendon collected from mice at different time points to further investigate the circadian-regulated proteome.

Results: Collagen I has a higher retention rate within the Golgi complex of VPS33b knockout (VPS33b KO) fibroblasts, and KO cells have a marked reduction in collagen fibril deposition. KO cells also have decreased contractility, and showed decreased amplitude in their circadian rhythm; however, there is no obvious change in the migratory function of VPS33b KO fibroblasts. Proteomics results identified other circadian-regulated VPS family members which may also be involved in extracellular matrix secretion and turnover.

Discussion: VPS33b is crucial for collagen I secretion and deposition of collagen I fibrils. In addition, the effects of VPS33b KO in fibroblast functionality and circadian rhythm suggest that VPS33b may have functions in pathways not related to trafficking, or that VPS33b-regulated protein trafficking is involved in regulating fibroblastic functions. Further analyses on the effects of VPS33b KO on uptake of pre-existing collagen fibrils, as well as the mechanisms of VPS33b-regulated collagen I secretion, are currently underway.

Lumican inhibits *in vivo* melanoma metastasis by altering matrix effectors and invadopodia markers

K. Karamanou[*,†,‡]; M. Franci[§]; M. Onisto[¶]; A. Passi[**]; S. Chakravarti[††]; I. Proult[*,‡]; D. Vynios[†]; S. Brézillon[*,‡]

[*]Université de Reims Champagne Ardenne, Laboratoire de Biochimie Médicale et Biologie Moléculaire, Reims, France; [†]Biochemistry, Biochemical Analysis & Matrix Pathobiology Research Group, Laboratory of Biochemistry, Department of Chemistry, University of Patras, Patras, Greece; [‡]CNRS UMR 7369, Matrice Extracellulaire et Dynamique Cellulaire, Reims, France; [§]Department for Life Quality Studies, University of Bologna, Rimini, Italy; [¶]University of Padova, Department of Biomedical Sciences Padova, Padova, Italy; [**]Dipartimento di Scienze Chirurgiche e Morfologiche, Università degli Studi dell'Insubria, Varese, Italy; [††]Cornea Division, Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, USA

Introduction: Lumican, a small leucine-rich proteoglycan, was reported to inhibit the membrane type matrix metalloproteinase MMP-14 activity and melanoma cell migration *in vitro* and *in vivo*. MMP-14 has been implicated in the migratory and metastatic potential of cancer cells. Moreover, Snail was reported to increase EMT and the metastatic potential of cancer cells. Therefore, the aim of this study was to analyse the effect of lumican on Mock and Snail-overexpressing B16F1 cells *in vivo*.

Materials and Methods: Intravenous injections of Mock-B16F1 and Snail-B16F1 cells ($n = 250 \times 105$ per IV) were performed in $Lum^{+/+}$ ($n = 24$) and in $Lum^{-/-}$ ($n = 24$) mice. At day 24, mice were sacrificed and lungs were collected. Apart from the in vivo experiments, in vitro methods, like confocal immunofluorescence, real-time PCR, and Western blots, were conducted, too.

Results: The number of metastatic nodules was significantly higher in mice injected with Snail-overexpressing B16F1 cells than in mice injected with Mock-B16F1 cells in both groups of mice. In addition, endogenous lumican of wild-type mice significantly inhibited the number of metastatic nodules as compared to lumican deleted mice. Moreover, *in vitro*, lumican inhibited the expression of cortactin (an invadopodia marker), CD44 (hyaluronan receptor), and heparanase. Thus, lumican is able to inhibit the expression of key molecules involved in cancer invasion and metastasis which might explain, at least in part, its inhibitory effect on lung metastatic nodules formation. In addition, the effect of lumican was observed *in vitro* in 3D invasion assays using scanning electron and confocal microscopy. Lumican was able to alter the formation of lamellipodia associated with a more rounded cell shape. Finally, lumican was shown to inhibit the phosphorylation of FAK, Akt, p130Cas and GSK3 α/β.

Discussion: Altogether, the results suggest that a lumican-based strategy targeting Snail-induced metastasis could be a useful therapeutic for melanoma treatment.

Dynamic expression of matrix components during skeletal development

S. Ahmed; N. C. Nowlan

Department of Bioengineering, Imperial College London, United Kingdom

Introduction: A dynamic and complex collagen network is essential for normal functioning of cartilage and bone. Understanding how this network emerges during development would be critically useful both for understanding the degeneration of skeletal tissues and for tissue engineering of replacement cartilage and bone. However, when and how the key collagens in cartilage and bone develop remains unclear. We examine how the structural organisations of collagen type I, II, III, V, VI and X emerge in the rudiments of the murine forelimb during prenatal development.

Materials and Methods: Mouse embryos were harvested and staged according to Theiler stages TS22, TS25 and TS27 (typically embryonic days 13.5, 15.5 and 17.5). Collagen distribution was studied with immunofluorescence and confocal microscopy.

Results: Initially, collagen I was expressed at the presumptive joint line (TS22 being prior to cavitation), and perichondrium. At TS25 it was expressed in the bone collar and by TS27, it was only observed at the site of endochondral ossification. Collagen II was consistently expressed in cartilage but was absent from mineralised and presumptive mineralised regions. At TS22-TS25, collagen II appeared mesh-like, but this patterning was less prominent at TS27. At all stages examined, collagen III was expressed throughout the rudiment including the mineralised cartilage with a pericellular expression. Collagen V was initially expressed throughout the diaphysis but became restricted to mineralised cartilage at TS25-TS27. At all stages examined, collagen VI was detected throughout the rudiment with a pericellular expression pattern. In the growth plate of TS25-TS27 rudiments, it was prominently stacked in rows and had a fibrilar distribution in the mineralised cartilage. At TS22, collagen X was expressed in the primary ossification centre. Later (TS25-TS27) collagen X distribution was only observed in the growth plates.

Discussion: All but collagen III had a dynamic expression in the developing skeletal rudiment, offering novel insights into when and how the key collagens in cartilage and bone develop. We propose that these dynamic expressions and compositional arrangements are critical to normal growth, morphogenesis and maturation of developing cartilage and bone, with implications of understanding the disease and regeneration of skeletal tissues.

The role of circadian clock genes in regulating the chondrogenic potential of human pluripotent stem cells

M. Naven; C. Smith; S. Kimber; Q.-J. Meng

Division of Cell Matrix Biology & Regenerative Medicine, University of Manchester, Manchester, M13 9PT, United Kingdom

Introduction: Osteoarthritis is a disease that results in the progressive loss of articular cartilage. Human pluripotent stem cells (hPSCs) differentiated towards a chondrocyte lineage may provide a scalable source of cells for a regenerative therapy. The circadian molecular clock is a fundamental component in the homeostasis of tissues such as cartilage. hPSCs do not appear to maintain a circadian clock until it is

activated at a certain point during differentiation. The aim of this study is to determine exactly how and when the cell intrinsic circadian molecular clock is activated during chondrogenic differentiation of hPSCs. Moreover, can modulation of the molecular clock be used to enhance chondrogenic outcomes?

Materials and Methods: hPSCs expressing the reporter mPer2::Luciferase were directed towards a chondrocyte lineage using a defined 14 day 2D differentiation protocol. After day 14, cells were cultured in 3D pellet culture for a further 28 days. Protocol cultures were assessed using bioluminescence microscopy at different points throughout the protocol to determine the point at which the circadian molecular clock is activated. In addition, a juvenile chondrocyte cell line (TC28a2), which displays an active circadian molecular clock, were cultured in 2D and 3D for comparison.

Results: Initial results show that circadian rhythm is indeed absent in hPSCs and is subsequently activated during the 3D phase of chondrogenic differentiation. In addition, the 3D culture phase of the differentiation protocol produces extracellular matrix (ECM) structures architecturally comparable to those seen in histological analysis of cartilage.

Discussion: The activation of the mPer2::luciferase reporter during the 3D stage of chondrogenic differentiation suggests that this model may be used to evaluate the currently unknown mechanisms leading to the activation of the circadian molecular clock. Moreover, the production of ECM structures comparable to those seen cartilage may be used to investigate the influences of circadian controlled gene transcription on pellet ECM composition.

Collagen is not just collagen—differential matrix expression induced by TGF-β and PDGFs

A. L. Reese-Petersen; M. A. Karsdal; F. Genovese

Nordic Bioscience

Introduction: Accumulation of extracellular matrix (ECM) proteins is the hallmark of fibrosis, which can lead to altered tissue homeostasis, organ failure and ultimately death. Many different cell types and growth factors are involved in this process but fibroblasts are the main source of ECM proteins. Here, we present results from an in vitro model indicating that transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF)-AB and BB induce synthesis of different ECM proteins relevant for pathogenesis.

Materials and Methods: The effect of TGF-β and PDGFs on ECM protein synthesis was assessed in a scar-in-a-jar (SiaJ) cell model using human renal fibroblasts. Cells were seeded in 48-well plates at 30 000 cells/well and incubated for 24 h in DMEM + 10% FBS. Serum starvation was done by seeding the cells for further 24 h in DMEM + 0.4% FBS. Fresh medium was added at day 0 with 225/150 mg/mL Ficoll 70/400 and 1% ascorbic acid, containing 0.04 nM TGF-β, 4-, 0.4-, or 0.04 nM PDGF-AB or -BB or a vehicle control. Medium was changed and collected at days 3, 6, 10 and 13. Biomarkers of collagen type I (PINP), III (PRO-C3), VI (PRO-C6) and fibronectin (FBN-C) formation were assessed in the medium.

Results: TGF-β induced a significant increase in PINP, PRO-C3 and FBN-C compared to PDGFs and the vehicle. Levels increased more than 50-fold for PINP and FBN-C and 10-fold for PRO-C3 compared to vehicle. PDGFs increased FBN-C and PINP but not PRO-C3 compared to the vehicle, but with lower potency than TGF-β. PRO-C6 was inhibited by TGF-β and dose-dependently stimulated by PDGFs. FBN-C and PINP peaked after 6 days of TGF-β treatment, while PRO-C3 and PRO-C6 peaked after 10 days of TGF-β and PDGF stimulation respectively.

Discussion: These data provide insight into the complex regulation of ECM protein synthesis induced by growth molecules and show that different growth factors induce different protein expression profiles in fibroblasts. Collagen synthesis is thus regulated differentially. This SiaJ model in combination with the investigated biomarkers of ECM formation could be used to elucidate the mechanisms behind acute and sustained matrix production profiles. Ultimately, this could point toward novel therapeutic intervention points in fibrogenesis.

Developing an *in vitro* human induced pluripotent stem cell (hiPSC) model using CRISPR/Cas9 to investigate the role of perlecan in fibrosis

J. Smith*; C. Merry*; M. Lord†; C. Denning*; J. Whitelock*,†

**Wolfson Centre for Stem Cells, Tissue Engineering and Modelling, University of Nottingham, Nottingham, United Kingdom, NG72RD; †Graduate School of Biomedical Engineering, University of New South Wales, Sydney, 2052, Australia*

Introduction: Perlecan is a modular heparan sulphate proteoglycan of basement membranes and the basal laminae. The N-terminal domain of the protein core, domain I, has three glycosaminoglycan attachment sites decorated with heparan sulphate. The C-terminal domain, domain V, contains a 12 integrin binding site and glycosaminoglycan attachment site that can be decorated with chondroitin sulphate. This domain has been implicated in the fibrosis associated with renal allograft transplantation, but the mechanism of action is not clear. Domain III has laminin-like domains and has been implicated in cell adhesion with the precise mechanism not yet elucidated. This project aims to use the CRISPR/Cas9 gene targeting system to specifically target these domains so that we can investigate their roles in fibrosis.

Materials and Methods: CRISPR/Cas9 targeting plasmids were designed and constructed with Snapgene™ using the genomic perlecan (*HSPG2*) sequences from the UCSC server (https://genome.ucsc.edu/) and PubMed™. Guide plasmids were designed using the CRISPR guide design site at MIT (http://crispr.mit.edu/). hiPSCs were transfected and clones selected through puromycin/blasticidin resistance. The expression of perlecan was assessed using immunocytochemistry and domain-specific antibodies together with qPCR and domain-specific primer sets.
Results: We have constructed a knockout plasmid targeting the start of exon 2 of *HSPG2* by introducing a premature stop codon. After transfection into hiPSCs and selection, we isolated heterozygous clones in which one allele was disrupted. These have reduced perlecan mRNA (~50% by qPCR), and protein expression by immunofluorescence. Targeted clones maintained pluripotency and capacity to differentiate into cardiomyocytes, demonstrating that a reduced expression of perlecan had no significant effect on the ability of hiPSCs to respond to differentiation cues.
Discussion: Our results show that we can use CRISPR/Cas9 gene targeting to successfully target the perlecan gene (*HSPG2*) to create clonal hiPSC lines with reduced expression, but maintain differentiation ability. Next, we plan to create a homozygous perlecan deletion and hiPSC lines that have been modified to express truncated forms of perlecan lacking either domain III or V. These cell lines will be used to investigate ECM composition and secretion as the cells differentiate into cardiomyocytes and respond to fibrotic stimuli.

Co-ordination of mechanotransduction by talin and vinculin

P. Atherton*; F. Lausecker*; I. Barsukov†; D. Critchley‡; C. Ballestrem*

*Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Manchester M13 9PT, United Kingdom; †Department of Molecular Cell Biology, University of Leicester, Lancaster Road, Leicester, LE1 9HN, United Kingdom; ‡Institute of Integrative Biology, University of Liverpool, BioSciences Building, Crown Street, Liverpool L69 7ZB, United Kingdom

Introduction: Cells continuously probe and respond to biochemical and mechanical properties of their surroundings. The extracellular matrix (ECM) is an important part of the cellular surrounding and cells sense it through transmembrane adhesion receptors (integrins). In focal adhesions (FAs), integrins are linked to the contractile actin cytoskeleton via a large number of FA plaque proteins that dynamically regulate this link. Of particular interest are the proteins talin and vinculin, which connect integrins to the actin cytoskeleton.
Materials and Methods: We use photokinetic microscopy (FLAP) to measure protein turnover within FAs on engineered polyacrylamide substrates of different stiffness. To investigate mechanisms of talin-vinculin interactions and how these contribute to mechanosensing, we targeted either protein to a force-free environment at the mitochondria, allowing us to study how forces contribute to protein activation.
Results: Our findings suggest that FAs are built of different modules: one comprising the mechanosensing adaptor proteins talin and vinculin; and the other containing signalling proteins such as FAK and paxillin. We show that the mechanosensing proteins are involved in rigidity sensing, whereas the signalling proteins control downstream signalling to influence the actin cytoskeleton. Using a number of talin and vinculin mutations, we demonstrate how they mutually interact, and show that active vinculin can bind to talin independently of forces, and *vice versa*. Mechanistically, this is facilitated by relief of talin auto-inhibition, which may act to destabilise the main vinculin-binding region with talin.
Discussion: Together, our data suggest that FAs are composed of functional modules that separately control the events of mechanotransduction, which involves both mechanosensing and mechanosignalling. Talin-vinculin interactions can occur independently of forces, although forces are required for mechanosensing.

Laminin-332 in the progression of cutaneous squamous cell carcinoma—3D cell culture system as an *in vitro* tumor model

E. Siljamäki*,†; P. Rappu†,‡; V. M. Kähäri*,†; J. Heino†,‡

*Department of Dermatology, University of Turku and Turku University Hospital, Turku, Finland; †Department of Biochemistry, University of Turku, Turku, Finland; ‡MediCity Research Laboratory, University of Turku, Turku, Finland;

Introduction: Cutaneous squamous cell carcinoma (cSCC) is the most common metastatic skin cancer. At present, no validated biomarkers are available for identification of cSCCs that progress to invasive or metastatic carcinomas, and thus, the prognosis of metastatic cSCC is poor. To investigate the progression of cSCC, we established a 3D cell co-culture model (spheroids) using human keratinocyte-derived cell lines and human primary skin fibroblasts.
Altered expression of basement membrane protein Laminin-332 has been linked to many carcinomas, including SCC. We show that in cancer cells, Laminin-332 expression is increased by fibroblasts, which are also crucial for cancer cell invasion. In addition, we connect Laminin-332 expression to oncogenic H-Ras signaling.
Materials and Methods: HaCaTs and H-Ras-transformed HaCaT human keratinocytes (A5, II-4 and RT3) were

cultured with or without human primary skin fibroblasts in 3D spheroids (3D Petri Dish, MicroTissues). Mass spectrometry, Western blotting and immunofluorescence stainings were performed to detect Laminin-332 expression in 3D spheroids. H-Ras was silenced by siRNA transfection. Invasion assays were conducted using collagen I gel (bovine skin collagen I, Nutragen).

Results: The organization of HaCaT/HaCaT-ras human keratinocytes reflected their tumorigenicity in 3D co-cultures with primary fibroblasts. Mass spectrometric analysis and Western blotting from the same co-cultures showed an increase in Laminin-332 expression compared to monocultured spheroids. Expression was dependent on oncogenic H-Ras, since H-Ras silencing in cancer cells completely blocked Laminin-332 expression in co-cultured spheroids. However, this did not occur in traditional 2D cell culture conditions. Additionally, cancer cell invasion from 3D spheroids occurred only when co-cultured with fibroblasts.

Discussion: Our data show that *in vitro* 3D cell culture model is a valuable tool for exploring the progression of cSCC. We demonstrate differential Laminin-332 expression in 2D and 3D cell cultures, highlighting the importance of more physiologically relevant, multicellular 3D cell culture models in cancer research. We show that Laminin-332 expression in cancer cells is considerably increased by a soluble factor released by primary skin fibroblasts, and the expression is linked to oncogenic H-Ras signaling. Additionally, the cancer cells invaded only in the presence of fibroblasts. Our results emphasize the indispensable role of ECM proteins and fibroblasts in cancer cell invasion and metastasis.

Perineuronal chondroitin sulfates reinforce inhibitory GABAergic transmission in the maturation of sensori-motor function

T. P. Y. Kwan*; D. K. Y. Shum*,†; Y. S. Chan*,†

School of Biomedical Sciences, The University of Hong Kong, Sassoon Road, Hong Kong, China; †State Key Laboratory of Brain and Cognitive Sciences, The University of Hong Kong, Sassoon Road, Hong Kong, China

Introduction: The last steps in the maturation of sensori-motor functions are often subject to experience-dependent modulations. In rats, we observed onset of negative geotaxis (an innate behavior indicative of graviception) as from postnatal day (P)9, time-matched with the consolidation of chondroitin sulfate (CS)-rich perineuronal nets (PN) around GABAergic/parvalbumin-expressing neurons in the vestibular nucleus (VN). Experience-dependent plasticity was revealed when bilateral labyrinthectomy in neonates significantly deterred both the onset of negative geotaxis and the consolidation of PN-CS around GABAergic interneurons in the VN.

Materials and Methods: Using SD rats as model, negative geotaxis and air righting were investigated as behavioural readout of graviception. Whole-cell patch-clamp recordings were performed on VN interneurons in brainstem slice at P9 and P14 to investigate spontaneous inhibitory and excitatory post-synaptic GABA or AMPA receptor-mediated current (sIPSC and sEPSC) and EPSC/IPSC ratio.

Results and Discussion: In labyrinth-intact rats, treatment of the VN at P6 with chondroitinase ABC (ChABC) resulted in loss of PN-CS. The effect lasted up to P13 during which developmental onset of negative geotaxis was delayed to P13. Is PN-CS critical for hardwiring inhibitory GABAergic transmission in the postnatal vestibular circuit? With whole-cell patch-clamp recordings performed on VN interneurons in brainstem slice preparations at P9 and P14 when PN-CS underwent consolidation, we found significant increase in frequency of sIPSC. Pre-treatment of the VN with ChABC at P6 led to 50% reduction in frequencies of IPSCs both at P9 and at P14 but no accompanying change in amplitude of sIPSC. After acute ChABC treatment, significant increase of EPSC/IPSC ratio for frequency but not for amplitude per sampled neuron was likewise observed. In contrast, neither frequency nor amplitude of sEPSC showed significant change in this period. Support is thus provided for a role of PN-CS in reinforcing inhibitory/GABAergic inputs to VN interneurons in the maturation of the vestibular pathway for the graviceptive behaviour.

Acknowledgement: The project is supported by HKRGC-GRF 17125115 and HKU Seed Funding for Strategic Interdisciplinary Research Scheme.

Characterization of AMACO and other basement membrane-associated Fraser complex proteins

T. Esho*; B. Kobbe*; T. Imhoff*; D. R. Keene†; R. Zent‡; M. Paulsson*; R. Wagener*

Center for Biochemistry, Medical Faculty, University of Cologne, 50931 Cologne, Germany; †Shriners Hospital for Children, Portland OR, USA; ‡Division of Nephrology and Hypertension, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA

Introduction: AMACO (VWA2 protein) is a basement membrane-associated protein containing VWA-like domains. It is expressed in skin, developing teeth, kidney, and choroid plexus, and was found as a molecular signature for hair placodes. AMACO is strongly expressed when invagination or budding occurs during development. The function of AMACO could be to mediate contact between epithelial and underlying mesenchymal cells. AMACO is part of the Fraser complex (FC). Fras1/Frem proteins each contain 12 CSPG (chondroitin sulphate proteoglycan) repeats and

thereby form a distinct subgroup of the Fraser complex proteins. Loss of expression of FC components like Fras1 or Frem proteins causes Fraser syndrome, in which cohesion between epithelial tissues and stroma is perturbed.

Materials and Methods: Frem1, -2 & -3 CSPG repeats were cloned using mouse cDNAs from embryo, brain, kidney and ovary. The recombinant protein secondary structures were established with circular dichroism experiment. Protein interaction was demonstrated by surface plasmon resonance and confirmed by ELISA style binding assays. The recombinant Frem2 protein was later used to produce an affinity purified antibody. We studied the architecture of the FC in embryonal skin by immunoelectron microscopy using gold-labelled antibodies.

Results: Frem2 and -3 CSPG repeats were successfully expressed in eukaryotic cells. While Frem3 CSPG repeats were retained in the cells, Frem2 CSPG repeats were however secreted into the supernatant and therefore produced on a large scale. The Frem2 CSPG repeats form predominantly a monomer, are properly folded and contain mainly β sheets. CSPG repeats of Frem2, like those of Fras1, also interact with AMACO. AMACO binds to the CSPG repeats of Fras1 and also to nephronectin. AMACO transiently forms previously unknown, extended cable-like suprastructures in the dermis that was observed also in human fetal skin (GW 21).

Discussion: AMACO and Frem2 were further characterized as members of the Fraser complex. Although they are expressed by keratinocytes, cables of about 60 nm originate at the dermal side of the basement membrane and often end at the cell surface of fibroblasts. The binding partners Fras1 and nephronectin have a more restricted distribution which only partially overlaps. We have recently started to characterize an AMACO KO mouse to study its function.

Investigating the roles of perlecan using antibodies with unique epitopes

Z. Elgundi*; A. Freelander*; P. Bean†; J. Whitelock*; M. Lord*

Graduate School of Biomedical Engineering, University of New South Wales (UNSW), Sydney, NSW, Australia; †Commonwealth Scientific and Industrial Research Organisation (CSIRO), North Ryde, NSW, Australia

Introduction: Perlecan is a major heparan sulfate proteoglycan of basement membranes. The core protein is divided into five domains with each domain involved in modulating various cellular processes through interactions with extracellular matrix molecules such as laminins as well as growth factors and cytokines. With such an integral role in regulating cell adhesion and proliferation to vascular development and immune function, perlecan is being studied for its role in tumorigenesis and its potential as a novel target for therapeutic intervention.

Materials and Methods: Using hybridoma technology, we have generated a panel of monoclonal antibodies that bind human perlecan. The binding specificity of the antibodies to full-length perlecan was confirmed by enzyme immunosorbent assay (ELISA) and Western blotting. The antibodies have been epitope binned using surface plasmon resonance (SPR) technology by screening the antibodies against recombinantly expressed perlecan domains. Having identified anti-perlecan antibodies with unique epitopes, the antibodies were investigated for their functional properties of modulating endothelial, fibroblast and cancer cell line adhesion, proliferation and migration in a series of cell-based assays.

Results: The antibodies have been organised into distinct bins based on their binding profile to individual domains and their ability to competitively block or sandwich pair with one another. Anti-perlecan antibodies that specifically bind to domains I, III and V have been identified. The binding specificity correlated with functional activity with domain-specific antibodies modulating or inhibiting adhesion, proliferation and migration of model cell lines used to reflect the main cell types localised in the tumour stromal environment including endothelial (HUVEC), fibroblast (MRC-5) and cancer (colon: WiDr) cells.

Discussion: These studies have demonstrated the significance of epitope mapping antibodies prior to the downstream assessment of functional activity. The binding specificity to perlecan domains combined with functional screening has provided insight into the functional relevance of the individual domains and their interacting molecules to elucidate the roles of perlecan in biology and pathology.

Fine structural modifications of heparan sulfate sulfation patterns in lung are associated with functional effects in precapillary pulmonary hypertension

P. Albanese*; S. J. Dumas†,‡; B. Ranchoux†,‡; S. Shamdani*; S. Chantepie*; M. B. Huynh*; D. Montani†,‡,§; B. Girerd†,‡,§; P. Dormuller†,‡; E. Fadel†,‡; P. Dartevelle†,‡; F. Perros†,‡; M. Humbert†,‡,§; D. Papy-Garcia*; S. Cohen-Kaminsky†,‡,§

ERL CNRS 9215, Laboratoire Croissance, Régénération, Réparation et Régénération Tissulaires (CRRET)/Université Paris Est Créteil, Université Paris Est, 94010, Créteil, France; †INSERM UMR-S 999, Hôpital Marie Lannelongue, 92350, Le Plessis-Robinson, France; ‡niv. Paris-Sud, Faculté de Médecine, Université Paris-Saclay, 94270, Le Kremlin-Bicêtre, France; §AP-HP Assistance Publique-Hôpitaux de Paris, Service de Pneumologie, Hôpital Bicêtre, 94270, Le Kremlin-Bicêtre, France

Introduction: Sulfated glycosaminoglycans (GAG), heparan (HS) and chondroitin (CS) sulfates, are essential components of the extracellular matrix (ECM) in lung homeostasis. In pulmonary hypertension (PH), altered ECM participates to lung

arterial remodeling. We hypothesized that changes in GAG patterns, composition and fine structure could alter their selective effects on growth factors binding and cell properties, thus leading to vascular remodeling in lung PH. Here, we deciphered the fine structure and bioactivity of GAGs both in human and rat experimental PH.

Materials and Methods: We performed quantitative (GAG total amount, HS/CS ratio), qualitative (disaccharidic sulfation pattern) and functional analysis (growth factor binding, cell proliferation) of HS and CS extracted from human lungs of patients with precapillary PH and controls, and of monocrotaline PH rats. Patients with idiopathic (iPAH), heritable (hPAH) PAH, or pulmonary veno-occlusive disease (PVOD) were included. Both human and animal studies were approved by ethical committees.

Results: Lungs of patients with iPAH, hPAH and PVOD revealed specific HS accumulation associated with selective HS disaccharides sulfation patterns. Monocrotaline rats presented increased GAGs/mg lung, preceding vascular remodeling, together with changes in disaccharides sulfation patterns, suggesting early alteration of GAG structure in disease progression. These changes were associated with significant functional modification of GAG binding affinity to growth factors (VEGF, FGF-2, PDGF), and significant induced cell proliferation in the presence of altered GAGs and growth factors.

Discussion: We suggest that changes of GAG composition and HS sulfation patterns in the lung could maintain a chronic inflammatory and vascular remodeling process leading to deregulated vascular cell proliferation through abnormal binding to growth factors. Further studies are focusing on new glycanic targets in pathological lung for future therapeutic intervention.

Modification of the extracellular matrix of the arterial wall by myeloperoxidase contributes to atherosclerosis

H. Cai; C. Y. Chuang; M. J. Davies

Department of Biomedical Sciences, Panum Institute, University of Copenhagen, Denmark

Introduction: The extracellular matrix (ECM) of the vascular basement membrane is vital for maintaining the functional and mechanical properties of arteries. The structure and properties of this material are altered during the development of atherosclerosis. The oxidant-generating enzyme myeloperoxidase (MPO) is known to be present in human atherosclerotic lesions, and the concentration of this enzyme correlate with disease severity and outcomes. This study examined the hypothesis that hypochlorous acid (HOCl), a powerful oxidant generated by MPO at micromolar concentrations, affects native human coronary artery smooth muscle cell (HCASMC)-derived ECM and that these modifications modulate HCASMC behaviour and phenotype, thereby contributing to progression of atherosclerosis.

Materials and Methods: Native HCASMC-derived ECM was harvested and exposed to increasing concentrations of HOCl (0-200 μM, both reagent and generated by MPO) and the effects on ECM proteins investigated using SDS-PAGE and antibodies against specific ECM proteins via ELISAs and Western blots. Modulation of HCASMC adhesion and proliferation was examined using calcein-AM and MTS. mRNA was isolated from HCASMC exposed to HOCl-modified ECM to investigate effects of modified ECM proteins on genes associated with inflammation, ECM protein synthesis and turnover, via quantitative real-time PCR.

Results: HCASMC-ECM exposed to increasing concentrations of HOCl (0-200 μM) resulted in changes in structure and a loss of antibody reactivity against the fibronectin cell-binding fragment (CBF), laminins, type IV collagen and versican G1 domain. Exposure of HCASMC to ECM pre-treated with > 10 μM HOCl, resulted in concentration-dependent reduction in HCASMC cell adhesion and proliferation. mRNA expression of HCASMC genes associated with the inflammatory response (*IL-6*, *COX-2*), matrix protein synthesis (*FN1*) and turnover (matrix metalloproteinases; *MMP1*, *MMP11*, *MMP13*), were up-regulated by ECM pre-treated with > 1 μM HOCl.

Discussion: These data show that HOCl induces structural and functional damage to HCASMC-ECM proteins, which subsequently can modulate HCASMC cell adhesion and proliferation. Our results reveal a novel pathway through which enzyme (MPO)-induced oxidation modifies ECM components, and contributes to behavioural switching of HCASMCs, a key process during the progression of atherosclerosis.

Influence of *Ruta graveolens* 9CH on murine melanoma progression

C. Fuselier*; C. Terryn†; A. Berquand‡; A. Bonnomet†; M. Molinari‡; L. Martiny*; C. Schneider*

**UMR MeDyC CNRS 7369 Reims, France; †Plateforme PICT, URCA, France; ‡LRN EA 4682, URCA, France*

Introduction: Dynamic and reciprocal interactions generated by the communication between tumor cells and their matrix microenvironment, play a major role in tumor progression. Adhesion of specific sites to matrix components associate to formation of membrane protrusions, allow tumor cells to move along a determined pathway. Today,

some conventional treatments for cancer are often unable to provide the expected response. In order to improve their effectiveness, there is a rising demand in complementary and alternative medicine. Among them, homeopathy is already known to have effects on some tumor cells, particularly the *Ruta graveolens* specific homeopathic strain. In this study, the mechanism of action of *Ruta graveolens* 9CH was studied about tumor process on a specific matrix environment, to explore new therapeutic approaches in cutaneous melanoma.
Materials and Methods: B16F1 and B16F10 murine melanoma cells were seeded on fibronectin and treated with *Ruta graveolens* 9CH. Cells dispersed migration and cells circularity were analyzed for 24 h using time-laps videos. Cells stiffness (atomic force microscopy), membrane structure changes (confocal microscopy using Laurdan fluorescent probe) and actin cytoskeleton (immunofluorescence assay) were explored.
Results: We demonstrated that *Ruta graveolens* 9CH treatment reduces significantly and sustainably B16 cells dispersed migration by 30% by altering development of cell protrusions. Moreover, *Ruta graveolens* 9CH greatly decreases cell stiffness on peripheral areas. Concomitantly, a disruption of actin filaments located just under the plasma membrane is observed from 1 h of treatment. Lastly, this homeopathic drug could alter plasma membrane structure by accumulating large ordered lipid domains.
Discussion: In conclusion, this study allowed us to demonstrate that *Ruta graveolens* 9CH disrupted *in vitro* murine melanoma cells migration by likely disrupting the balance between ordered and disordered lipid domain of plasma membrane. Whereas the correlation between lipid raft and cytoskeleton disrupting is not well established, *Ruta graveolens* 9CH may act on actin cytoskeleton organization, as evidenced by cell stiffness decrease, which ultimately fails to establish an effective migration process.

ADAM12-mediated shedding of basigin controls the subcellular localization and lactate transport of MCT4 and enhances collagen degradation in cancer cells

R. Albrechtsen*; N. J. Wewer Albrechtsen†; M. Kveiborg*

*Biotech Research and Innovation Centre (BRIC), University of Copenhagen, Denmark; †Department of Biomedical Sciences and the Novo Nordic Foundation Centre for Basic Metabolic Research, University of Copenhagen, Denmark

Introduction: Basigin (also named CD147/EMMPRIN), a transmembrane glycoprotein of the immunoglobulin superfamily, regulates the localization of monocarboxylate transporters (MCT)-1 and -4 at the cell surface, which allow the efflux of lactate produced by aerobic glycolysis. Moreover, basigin stimulates matrix metalloproteinase (MMP)-mediated extracellular matrix (ECM) degradation, driving cancer cell invasion. Basigin can be proteolytically shed from the cell surface and serum levels of soluble basigin in cancer patients often correlates with disease stage and poor prognosis. Interestingly, a positive correlation between basigin and a disintegrin and metalloprotease (ADAM)-12 in serum from prostate cancer patients was recently reported. Yet, the functional relevance of this correlation is unknown.
Materials and Methods: ADAM12 was overexpressed or knocked down in human cancer cells and basigin shedding was assessed by Western blot. Immunofluorescent staining and microscopic analysis were used to determine protein localization and gelatin degradation. Database mining was performed to correlate gene expression and identify cancer-associated mutations.
Results: ADAM12 interacts with basigin and cleaves it in the juxtamembrane region. Overexpression of ADAM12 in human cancer cells induces endogenous basigin shedding, enhances the amount of MCT4 at the cell surface, and causes a significant increase in lactate efflux. Highlighting the relevance of this finding, RNAseq data show a statistically significant correlation between the expression of ADAM12 and MCT4 in human prostate tumors. Further database mining identified several cancer-associated point mutations in the basigin membrane proximal region. Interestingly, expression of identified basigin mutants in human cells showed that several of these are highly prone to ADAM12-mediated shedding and therefore more potently enhance the gelatinase activity of exposed cancer cells.
Discussion: ADAM12-mediated shedding of basigin may regulate the sub-cellular localization of MCT4 and consequent lactate efflux in cancer cells. Moreover, basigin shedding appears to be altered in some types of cancer, with potential implications for the ability of cancer cells to degrade the ECM and invade surrounding tissues.

Vascular chondroitin/dermatan sulfate proteoglycans remodeling induced by ApoA-I and natural variants. Probable role in settlement of amyloidosis

A. C. Eiguren*; F. Funez*; A. M. Birocco*; S. Rosu†; A. Tricerri†; G. C. Calabrese*

*Universidad de Buenos Aires. Facultad de Farmacia y Bioquímica. Departamento de Ciencias Biológicas. Cátedra de Biología Celular y Molecular. Junín 954, Primer Piso, (C1113AAD) Buenos Aires, Argentina; †Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CONICET, La Plata, Buenos Aires, Argentina

Introduction: Amyloidosis constitutes a heterogeneous group of diseases involving protein misfolding and deposition of fibrils. Apolipoprotein A-I (apoA-I), the main protein of plasma

high-density lipoproteins (HDL), removes excess cell cholesterol and protects against atherosclerosis. Nevertheless, some natural variants (R173P) or their N-terminal fragments (IOWA, N-terminal peptide of G26R) of the native protein with structural disorder elicit their propensity to suffer misfolding or aggregation. Moreover, amyloidosis due to the protein with the native sequence has been described as diffuse protein aggregates in atherosclerotic plaques. Our previous reports suggest that specific interactions of apoA-I with glycosaminoglycans could elicit its retention and/or aggregation. Furthermore, recent studies indicate that protein cores of proteoglycans (PGs) may influence the type and modification patterns of the subsequently attached glycosaminoglycan chains. We hypothesize that mutations in human apoA-I may affect the core protein pattern expression of vascular chondroitin/dermatan sulfate PGs, modulating chemical changes in the glycosylation pattern which elicit extracellular apoA-I aggregation.

Materials and Methods: WT apoA-I and the amyloidogenic mutants IOWA and R173P were obtained by molecular biology techniques. Human umbilical vein endothelial cells (HUVEC) were treated with 1.5-50 μg/mL for 24 hr. MTT, immunofluorescence of NFκB and zymographic analysis were used to evaluate endothelial activation. PG protein cores were quantified using RT-PCR for decorin, biglycan and versican.

Results: WT, R173P or IOWA (1.5 μg/mL) treatment did not modify cell viability, NFκB nuclear translocation and metalloproteinase-2 and -9 activities. Decorin expression was significantly decreased by WT and R173P, 10- and 6-fold respectively, when it was compared with non-treated cells (control), whereas biglycan was increased 4-fold by IOWA variant. And versican expression was only detected after R173P treatment.

Discussion: WT and the studied natural variants do not elicit an inflammatory response in our experimental model. Nevertheless, our results indicate substantial modifications in the profile of chondroitin/dermatan sulfate PGs core proteins, depending on the protein or peptide employed. Considering that glycosaminoglycan polymerization and modification by sulfation and epimerization are influenced by the protein core, changes in the profile of PGs might be involved directly or indirectly in the equilibrium between protein function and cytotoxicity.

Development of human antibody against ADAM28, a key modulator of tumor microenvironmental factors in non-small cell lung carcinomas

S. Mochizuki*,†; M. Shimoda†; H. Ueno*; Y. Okada†,‡

Department of Surgery, National Defense Medical College; †Department of Pathology, Keio University School of Medicine; ‡Department of Pathophysiology for Locomotive and Neoplastic Diseases, Juntendo University, School of Medicine

Introduction: ADAM28 (a disintegrin and metalloproteinase 28) is overexpressed by carcinoma cells in non-small cell lung carcinomas (NSCLCs) and plays a central role in cancer cell proliferation and progression by regulating tumor microenvironmental factors in cancers. To aim for new target therapy of NSCLC patients, we have developed and characterized human neutralizing antibodies against ADAM28.

Materials and Methods: The Human Combinatorial Antibody Library (HuCAL) was used for the screening of antibodies. Specificity of antibodies was analysed by immunoprecipitation, immunoblotting, epitope mapping and Biacore system. Effects of the antibody on cell proliferation and apoptosis in lung adenocarcinoma cell lines were examined by BrdU incorporation and DNA fragmentation ELISA, and IVIS imaging system was used to test the effects on lung metastasis in NOD/SCID mice.

Results: By screening HuCAL using phage display panning, we obtained two antibodies 211-14 and 211-12, which inhibited the activity of ADAM28. Antibody 211-14 recognized the junctional region between the cysteine-rich domain and the secreted-specific domain with a K_D value of 94.7 pM. Proliferation of lung adenocarcinoma cell lines with ADAM28 expression such as PC-9 cells was effectively inhibited by the antibody, but no effect was observed on H1975 cells with negligible ADAM28 expression. von Willebrand factor-induced apoptosis was promoted by the antibody treatment only in ADAM28-expressing cell lines. In mouse lung metastasis models, antibody 211-14 significantly reduced tumor growth and metastases of PC-9 cells and prolonged survivals in the antibody-treated mice compared with the control IgG-treated ones. Combination therapy of the antibody and docetaxel was more effective than that of bevacizumab (human anti-VEGF antibody) and docetaxel, and showed further elongation of survival time compared with monotherapy. No adverse effects were observed even after administration 10-fold more than effective dose of the antibody to normal mice or monkeys.

Discussion: Our data demonstrate that antibody 211-14 is a neutralizing antibody specific to ADAM28 and suggest that this antibody may be a useful treatment remedy for NSCLC patients.

Binding stoichiometry of HSP47 to collagen depends on the collagen sequence

E. Abraham; U. Baumann; J. M. Gebauer

Institute of Biochemistry, University of Cologne, Cologne, Germany

Introduction: HSP47—also known as SERPINH1—is of outmost importance for the proper folding of collagen molecules and mutations in HSP47 can lead to defective collagen

secretion and *Osteogenesis imperfecta*. On the other side in cases of excessive collagen secretion (eg, fibrotic diseases) HSP47 was always found to be upregulated.

HSP47 was previously reported to recognise a specific motif on collagen helices (Gx[S/T]GxRGxx). Structural analysis of the collagen-HSP47 complex showed the importance of Asp_{385} which forms a salt bridge with the arginine on the collagen chain.

We identified a previously unknown high affinity binding site for HSP47 which introduces an additional phenylalanine in the binding motif (Gx[S/T]GxRG**F**x) [unpublished]; however, the exact mode of binding to this altered binding site was unknown.

Materials and Methods: We recombinantly expressed canine-derived HSP47 and foldon peptides containing a high affinity binding sequence. The binding properties were tested using an ELISA-style-binding assay and bio-layer interferometry. To understand our findings structurally, we co-crystallised HSP47 with the corresponding collagen mimetic peptides.

Results: Binding assays showed an increased affinity for peptides having a GxRG**F** motif, and slight improvement for GxRG**L** motifs. Interestingly, modelling phenylalanine in our existing crystals structure led to server steric clashes. Therefore, we successfully crystallised HSP47 in complex with synthetic, homotrimeric collagen peptides containing RGF and RGL sequences. Interestingly, both complexes crystallised in different crystals forms but as a 1:1 complex (collagen triple helix: HSP47), contrasting to our earlier structures showing a 1:2 complex.

Discussion: Preliminary analysis of the structures showed that HSP47 undergoes some minor rearrangements to form a pocket for the hydrophobic phenylalanine residue. The increased affinity most likely results from a gain of water entropy upon shielding of the aromatic residues. Interestingly, due to the staggered nature of the collagen helix, the bulky phenylalanine only allows a single HSP47 binding. At the second binding site, the phenylalanine residue is positioned differently and cannot be accommodated in the above-mentioned pocket. This demonstrates that although HSP47 is in principle able to bind on two sides, the actual stoichiometry greatly depends on the collagen sequence.

Kindlin-1 regulates the mammary tumour cell secretome

A. Byron; J. Ward; H. Patel; J. Li; S. Sarvi; G. L. Dodd; M. Muir; A. H. Sims; V. G. Brunton

Cancer Research UK Edinburgh Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, United Kingdom

Introduction: The focal adhesion protein kindlin-1 has been shown to regulate TGFβ-induced epithelial-to-mesenchymal transition in breast cancer cell lines, which has been attributed to increased invasive capacity. Indeed, increased expression of kindlin-1 has been associated with increased risks of lung metastasis in breast cancer, but the mechanistic basis of this link is unclear.

Materials and Methods: To investigate further the role of kindlin-1 in metastasis, the kindlin-1 gene, *Fermt1*, was deleted from mouse mammary tumour cells using the CRISPR-Cas9 system. Isolated secreted proteins were analysed by label-free mass spectrometry and bioinformatic approaches. Integrin-dependent cell adhesion was measured on defined substrates and endothelial cells. Experimental metastasis assays were performed in a mouse model of mammary tumourigenesis in the presence of inhibitors of integrin-mediated adhesion.

Results: Proteomic analyses of extracellular proteins identified a network of kindlin-1-dependent secreted proteins that were linked to metastasis. Interrogating multiple patient cohort datasets, survival analyses of the kindlin-1-dependent secretome genes demonstrated that they were significantly more associated with lung metastasis than with other (non-lung) metastasis. The secretome network included the large glycoprotein tenascin-C, a lung metastasis regulator associated with poor overall survival of patients with breast cancer. Regulation of tenascin-C expression was dependent on the ability of kindlin-1 to bind and activate β integrins, and β1 integrin was required for the outgrowth of micrometastases. Furthermore, loss of kindlin-1 expression reduced pulmonary arrest and metastatic colonisation in an integrin-dependent manner.

Discussion: These results show that the kindlin-1-dependent secretome is associated with lung metastasis and is elevated in cells metastatic to lung. Kindlin-1 may, therefore, contribute to a metastatic niche in pulmonary metastasis. Kindlin-1 expression enhances the metastatic potential of breast cancer cells by modulating integrin activity and promoting tumour cell adhesion to the metastatic niche. Thus, kindlin-1 is a critical mediator of early lung metastasis of breast cancer.

Using bioprinting to investigate axon pathfinding for peripheral nerve regeneration

A. Kolmogorova*; A. Reid†,‡; C. Ballestrem*; B. Derby§

**Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Manchester; †Blond McIndoe Laboratories, University of Manchester, Manchester; ‡University Hospital South Manchester, Southmoor Road, Manchester, United Kingdom; §School of Materials, University of Manchester, Manchester*

Introduction: Neuronal networks are critical for many body processes, such as brain function, movement and sensing. These neural networks are established during development

and depend on precise axon guidance mediated by extrinsic molecular cues and extracellular matrix (ECM) proteins. Following spinal cord (SCI) or peripheral nerve injury (PNI), axon regrowth is impaired, leaving individuals with compromised movement or, in case of SCI, paralyzed. Our key objective is to elucidate how different ECM proteins and chemical cues determine axon outgrowth and pathfinding, thereby permitting the development of novel biochemical micropatterned materials for enhanced nerve regeneration.

Materials and Methods: To examine neuronal behaviour under various conditions, inkjet printing, light-induced photopatterning (from Alveole) and microstamping techniques were compared to create an *in vitro* neuronal guidance assay. Light-induced photopatterning revealed to be the most precise and flexible tool to create patterns.

We produced Fibronectin (FN) and Laminin (LM) crossed line patterns as a competition assay. We used rat dorsal root ganglion (DRG) as a model system for peripheral nervous system (PNS) regeneration, and a mouse CNS-derived catecholaminergic neuronal cell line (CAD) as a model of the central nervous system (CNS).

Results: Our results indicate that defined tracks of FN and LM are able to guide axons of CAD cells and influence their directionality. Additionally, axons that started on FN preferred to stay on FN which could indicate a preference for FN. This could be due to integrin recycling or a protein concentration-dependent effect.

Furthermore, by establishing an algorithm for neurite tracking, data output was automated to give indication for directionality preferences. By performing live cell imaging technique, we will furthermore access the growth dynamics and speed of growth on these defined patterns.

Discussion: We showed a technique to create defined protein patterns by using light-induced photopatterning as a tool for axonal pathfinding assays. Future work includes testing different ECM proteins and guidance cues for their guidance capabilities on neurons from the PNS and CNS.

Knowledge about guidance abilities of defined biochemical cues will allow the design of micropatterned surgical nerve repair devices for *in vivo* testing.

The potent inflammatory oxidant, peroxynitrous acid, modifies basement membrane laminins in human atherosclerotic lesions

C. Y. Chuang; L. Lorentzen; P. Hägglund; M. J. Davies

Panum Institute, BMI, University of Copenhagen, Denmark

Introduction: The vascular basement membrane is rich in laminin, a trimeric extracellular matrix (ECM) protein consisting of α, β and γ chains. The C-terminus of the α chain interacts with specific integrins on cells and plays a critical role in cell adhesion and signalling. Binding sites for other ECM species including perlecan, collagens, fibronectin and nidogen are also present in specific domains. These interactions are perturbed in atherosclerosis, where activated neutrophils, monocytes and macrophages generate oxidants, including peroxynitrous acid (ONOOH), which can alter ECM composition and may contribute to endothelial cell (EC) dysfunction, ongoing inflammation and smooth muscle cell (SMC) infiltration and proliferation. We hypothesise that specific laminin isoforms synthesized by EC and SMC are important in maintaining an intact and functional ECM in healthy arteries and that inflammation and ONOOH formation modify laminin domains in a manner that alters cellular behaviour and promotes atherosclerosis.

Materials and Methods: Laminin isoforms synthesized by primary human coronary artery endothelial (HCAEC) and smooth muscle (HCASMC) cells and present in advanced human atherosclerotic lesions were characterized by SDS-PAGE and Western blotting. Structural changes to laminins induced by ONOOH were determined using ELISA, WB, UPLC and LC-MS. Effects of ONOOH on cell behaviour were examined using cell adhesion assays.

Results: The laminin isoforms generated by HCAEC or HCASMC, and present in their ECM, differ to those detected in advanced human atherosclerotic lesions. ONOOH-modified laminin was detected in advanced human lesions. Treatment of HCAEC or HCASMC ECM with ONOOH resulted in a $> 50\%$ loss of antibody recognition of the cell-binding epitopes, a dose-dependent loss of Tyr and Trp residues, and formation of the specific biomarkers, 3-nitroTyr, 6-nitroTrp and di-Tyr. These modifications have been mapped to specific sequences using LC-MS peptide mapping, with some present within functional domains. These changes were associated with a loss of HCAEC adhesion to the modified laminins.

Discussion: These data suggest that the laminin isoforms produced by HCAEC or HCASMC play specific roles in maintaining a functional and intact ECM environment in healthy arteries and that this balance is perturbed in atherosclerosis as a result of ONOOH formation. These laminin modifications appear to have functional consequences for cell behaviour.

Collagen prolyl 4-hydroxylases in collagen fibril formation and wound healing in mouse skin

K. Drushinin*,†; A. Salo*,†; I. Miinalainen*; R. Sormunen*; J. Myllyharju*,†

**Biocenter Oulu, University of Oulu, Finland; †Faculty of Biochemistry and Molecular Medicine, University of Oulu, Finland*

Introduction: One central enzyme in collagen biosynthesis is collagen prolyl 4-hydroxylases (C-P4Hs), which hydroxylates prolines in collagenous repetition domain (-Gly-X-Pro-)

to 4-hydroxyproline. Vertebrate C-P4Hs are $\alpha_2\beta_2$ tetramers with three isoforms of the catalytic α-subunit, β-subunit being always a protein disulfide isomerase. Knockout of *P4 ha1* gene is embryonically lethal, whereas knockout of *P4 ha2* gene causes no obvious phenotype. First recognized patient mutations in P4HA1 cause congenital connective tissues disorder. Here, we have studied the role of C-P4H-I and C-P4H-II in skin and cutaneous wound healing.

Materials and Methods: Mutant mice (P4 $ha1^{+/+}$; P4 $ha2^{+/-}$, P4 $ha1^{+/-}$; P4 $ha2^{+/-}$, P4 $ha2^{-/-}$, P4 $ha1^{+/-}$; P4 $ha2^{-/-}$) were used in the study. Skin structure and collagen fibrils were studied with histological stainings and transmission electron microscopy (TEM). The thickness of dermis was measured, and the amount and thermostability of the skin collagen was determined. The diameter of collagen fibrils in the dermis was measured. The wound healing was studied by following the closure and inflammatory response of cutaneous wounds and the skin inflammatory response was further studied by inducing inflammation with TPA and analyzing the inflammatory cells and cytokine levels.

Results: The skins of P4 $ha2^{-/-}$ and P4 $ha1^{+/-}$; P4 $ha2^{-/-}$ mice revealed a thinner dermis, less collagen and decreased collagen fibril diameter compared to control mice. Also, capillary basement membranes were expanded in these mice. The melting temperature of P4 $ha1^{+/-}$; P4 $ha2^{-/-}$ mice skin collagen was decreased. The wound healing study showed no differences in the speed of wound closure, but there was a stronger inflammatory response with P4 $ha1^{+/-}$; P4 $ha2^{-/-}$ mice in the beginning of the healing process. TPA-treated skins of P4 $ha1^{+/-}$; P4 $ha2^{-/-}$ mice had stronger inflammatory response.

Discussion: The decreased enzyme activity causes decreased collagen fibril thickness and collagen amount and alterations in the capillary basement membranes in the skin. In addition, the inflammatory response is affected. Thus, our results show the central role of C-P4Hs in thermostable collagen fibril formation, tissue organization and ECM function in inflammatory response.

Laminin α4 deletion leads to impaired hematopoietic regeneration following irradiation-induced injury and accelerates the progression of acute myeloid leukemia

M. Kondo*; P. Xiao*; L. Sandhow*; M. Dolinska*; T. Bouderlique*; A.-S. Johansson*; M. Ekblom†; H. Qian*

Center for Hematology and Regenerative Medicine (HERM), Department of Medicine Huddinge, Karolinska University Hospital, Karolinska Institute, SE-141 86 Stockholm, Sweden; †Division of Molecular Hematology, Lund University, Lund, SE-22184, Sweden

Introduction: Both normal hematopoiesis and leukemia are regulated by a specialized microenvironments in bone marrow (BM), termed as hematopoietic stem cell (HSC) niches, which consist of cellular niche components, extracellular matrix proteins (ECM), growth factors and cytokines. While our knowledge of HSC niche in hematopoiesis homeostasis has been advanced, the impact of the niche, in particular ECM, on hematopoiesis regeneration under pathological conditions remains poorly understood.

Laminins are heterotrimeric ECM composed of α, β, and γ chains and are present in BM. The laminin α4 chain (LAMA4) is an active component for LAMA4-containing isoforms. It has been reported that the interactions of LAMA4 with their receptor integrin α6 are important for HSC homing. However, the role of LAMA4 in regeneration after hematopoiesis injury and during leukemia development is not known.

Materials and Methods: We here have used *Lama4*$^{-/-}$ mouse model, multi-color flow cytometry, confocal imaging, and transplantation as well as an acute myeloid leukemia (AML) mouse model to investigate the impact of Lama4 loss on HSC niche maintenance and hematopoietic regeneration post-injury and during AML progression.

Results: Lama4 deletion resulted in the reduction of mesenchymal progenitor cells (MPC, $CD45^-TER119^-CD31^-CD44^-SCA1^-CD51^+$) and endothelial cells, but not mesenchymal stem cells (MSC, $CD45^-TER119^-CD31^-CD44^-SCA1^+CD51^+$) in adult mouse BM. Following sub-lethal irradiation, *Lama4*$^{-/-}$ mice displayed slower and incomplete recovery of platelets, mature myeloid cells and erythrocytes, which was accompanied with the reduced frequency of myeloid progenitors and the accumulation of megakaryocyte-erythrocyte progenitors and immature erythrocytes in the BM. These data suggest an important role of LAMA4 for hematopoiesis regeneration and megakaryocyte and erythrocyte maturation post-irradiation. Mechanistically, the delayed hematopoietic recovery might be associated with downregulation of *Il6* in the MSC and *angiopoietin-1* in the MPC of *Lama4*$^{-/-}$ mice. Most importantly, by transplanting AML cells with MLL-AF9 fusion gene into non-conditioned *Lama4*$^{-/-}$ mice, we demonstrated that *Lama4* deletion in BM niche led to an earlier onset of AML. Moreover, loss of *Lama4* in BM microenvironment accelerated AML relapse after therapeutic hematopoietic cell transplantation.

Conclusion: Altogether, our study suggests that LAMA4 is required for efficient hematopoietic recovery post–irradiation-induced injury and inhibits AML progression.

Syndecan-1: a new component of epithelial podosomes

M. Montmasson; A. Michopoulou; G. Dayan; E. Lambert; P. Rousselle

Laboratoire de Biologie Tissulaire et d'Ingénierie Thérapeutique, Lyon, France

Introduction: During the epithelialization phase of wound repair, basal keratinocytes migrate, proliferate and

maintain dynamic interactions with extracellular matrix (ECM). Laminin 332, known as a major adhesion substrate for keratinocytes, contributes to skin re-epithelialization through its α3 chain C-terminal globular domains 4 and 5 (so-called LG45). Recent studies have suggested that LG45 induces expression of the pro-migratory matrix metalloproteinase MMP-9. As syndecan-1 was shown to participate in cytoskeleton dynamic through binding to the laminin LG45 domains, we analysed its potential involvement in MMP-9 expression.

Materials and Methods: Site-directed mutagenesis was applied to alter binding properties of a recombinant LG45 protein. PCR and gel zymography approaches were used to analyze MMP-9 expression in the conditioned medium and ECM of keratinocytes. Syndecan-1 expression was knocked down with siRNAs in human primary keratinocytes. In situ gelatin zymography was assessed and analysis of various labelled antigens was done by confocal microscopy.

Results: Our PCR analysis and zymography results revealed that syndecan-1 plays a role in LG45 induced MMP-9 expression and activation. Down-regulating syndecan-1 expression in keratinocytes confirmed these findings and revealed that this phenomenon also occurred when cells were treated with TNFα or IL1β, two cytokines known to up-regulate MMP-9 expression. In situ zymography performed with primary keratinocytes revealed areas of digested gelatin resembling adhesion contacts underneath keratinocytes. Their number was increased in LG45-treated keratinocytes and their formation inhibited by MMP-9 inhibitors. Their deeper analysis by confocal microscopy revealed syndecan-1 staining as bright rings surrounding a core of actin, cortactin, ARP2/3 and WASP localized within the digested gelatin. These data suggest that these clusters belong to epithelial podosomes.

Discussion: Our data demonstrate for the first time that syndecan-1 belongs to epithelial podosomes and that its expression within their outward ring is required for their formation and the subsequent MMP-9 activity. Our data further reveal that the laminin LG45 domains increase their number and MMP-9 activity in a manner comparable to that of IL1β. Syndecan-1 distribution in filopodia at the front edge of migrating keratinocyte may have a role to play in the regulation of MMPs activity, therefore facilitating their path to regenerate the epidermal compartment.

Characterization of inflammatory breast cancer: a vibrational microspectroscopy and imaging approach at cellular and tissue level

H. T. Mohamed*; G. D. Sockalingum†; V. Untereiner‡; I. Proult§,¶; S. A. Ibrahim*; M. Götte**; M. El-Shinawi††; M. M. Mohamed*; S. Brézillon§,¶

*Department of Zoology, Faculty of Science, Cairo University, Egypt; †BioSpecT – BioSpectroscopie Translationnelle, EA7506, Université de Reims Champagne-Ardenne, UFR de Pharmacie, Reims, France; ‡Plateforme d'imagerie cellulaire et tissulaire (PICT), Université de Reims Champagne-Ardenne, Reims, France; §CNRS UMR7369, Matrice Extracellulaire et Dynamique cellulaire (MEDyC), Reims, France; ¶Université de Reims Champagne-Ardenne, Laboratoire de Biochimie Médicale et de Biologie Moléculaire, UFR de Médecine, Reims, France; **Department of Gynecology and Obstetrics, Münster University Hospital, Münster, Germany; ††Department of General Surgery, Faculty of Medicine, Ain Shams University, Egypt

Introduction: Inflammatory breast cancer (IBC) has a poor prognosis because of the lack of specific biomarkers and late diagnosis. An accurate and rapid diagnosis implemented early enough can significantly improve disease outcome. Vibrational spectroscopy has proven useful for cell and tissue characterisation based on intrinsic molecular information. Here, we have applied infrared and Raman microspectroscopy and imaging to differentiate between non-IBC and IBC at both cell and tissue levels.

Materials and Methods: Two human breast cancer cell lines (MDA-MB-231 and SUM-149), 20 breast cancer patients' (10 non-IBC and 10 IBC), and 4 healthy volunteers' biopsies were investigated. Fixed cells and tissues were analyzed by FT-IR microspectroscopy and imaging, while live cells were studied with Raman microspectroscopy. Spectral data were analyzed by hierarchical cluster analysis (HCA) and common K-Means algorithms.

Results: For both cell suspensions and single cells, FT-IR spectroscopy showed sufficient high inter-group variability to delineate MDA-MB-231 and SUM-149 cell lines. Most significant differences were observed in the spectral regions of 1096-1108 and 1672-1692 cm^{-1}. Analysis of live cells by Raman microspectroscopy gave also a good discrimination of these cell types. The most discriminant regions were 688-992, 1019-1114, 1217-1375 and 1516-1625 cm^{-1}. Finally, K-Means Cluster analysis of FT-IR images allowed to delineate non-IBC from IBC tissues.

Discussion: This study demonstrates the potential of vibrational spectroscopy and imaging to discriminate, without dewaxing, between non-IBC and IBC at both cell and tissue levels. A new concept based on spectral histology identifies non-IBC and IBC tissues in a label-free manner showing promises for diagnosis of breast cancer.

A *Col9a3* Exon 3 skipping mouse as novel model for multiple epiphyseal dysplasia

S. Lecci; C. Miles; P. Cairns; M. D. Briggs; D. A. Young

Institute of Genetic Medicine, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 3BZ, United Kingdom

Introduction: Multiple epiphyseal dysplasia (MED) is an autosomal chondrodysplasia characterized by early-onset degenerative joint disease and epiphyseal hypoplasia. Its

genetic background is complex and heterogeneous since mutations in several genes coding for extracellular matrix (ECM) components have been identified in patients. Among these genes are those encoding for the pro-α chains of the Type IX collagen, COL9A1, COL9A2 and COL9A3 where the majority of mutations lead to the skipping of exon 3.

Materials and Methods: By CRISPR/Cas9 technology, we generated a mouse carrying a deletion of *Col9a3* exon 3 ($Col9a3^{\Delta ex3}$), reproducing the splicing events reported in a MED patients group. Initial phenotyping of 3- and 9-week-old $Col9a3^{\Delta ex3}$ mice is underway including skeletal X-ray and growth plate analysis (through immunohistochemistry and BrdU labelling to monitor chondrocyte proliferation).

Results: CRISPR/Cas9 injection generated mice with the differing genomic deletion of exon 3, the breakpoints of which were confirmed by Sanger sequencing. The phenotyping of offspring through DNA and cartilage RNA analysis had led to the establishment of two transgenic mouse lines, one splicing as predicted ($Col9a3^{\Delta ex3}$) and a second almost completely lacking the Col9a3 transcript ($Col9a3^{-/-}$). Immunoblotting confirmed the lack of collagen type IX protein from predicted null mouse cartilage. Both lines are viable; however, only $Col9a3^{-/-}$ mice displayed detectable phenotypic abnormalities: mild short stature and hip dysplasia, abnormal tibial epiphysis morphology and reduced level of chondrocyte proliferation in a disorganized growth plate structure. No overt phenotype detected instead in $Col9a3^{\Delta ex3}$ line.

Discussion: Having confirmed the production of a shorter RNA from cartilage of $Col9a3^{\Delta ex3}$ mice, lacking only exon 3 and therefore splicing as expected and producing collagen type IX protein, no overt phenotype was detected; therefore, the future work will be focused on assessing their cartilage stability. $Col9a3^{-/-}$ line, instead, has a mild skeletal phenotype and shows no transcript and protein, results confirmed by WB and immunohistochemistry of the growth plate.

Both mutant mice will represent an important tool to gain insights on collagen IX structure and its role into the matrix. In particular, the exon skipping line, by recapitulating human Col9-MED, can add to our understanding of the disease mechanism responsible for the onset of MED.

Overexpression of Fibulin-7 modulates the Ang1-Tie2 system and contributes to the aberrant vasculature in glioblastoma

S. de Vega*; A. Kondo†; M. Suzuki†; E. Arikawa-Hirasawa‡; Y. Okada*

**Department of Pathophysiology for Locomotive and Neoplastic Diseases, Juntendo University Graduate School of Medicine, Tokyo, Japan; †Department of Neurosurgery, Juntendo University Graduate School of Medicine, Tokyo, Japan; ‡Research Institute for the Diseases of the Old Age, Juntendo University Graduate School of Medicine, Tokyo, Japan*

Introduction: Glioblastoma multiforme (GBM) is characterized by high invasiveness and hypervascularity, the latter of which is distinct from normal vasculature, showing hypertrophied and glomeruloid vessels. This complex vasculature involves an active remodeling of the extracellular matrix (ECM) by production of new ECM molecules and degradation of the existing ones. Fibulin-7 (Fbln7), an ECM protein of the Fibulin family, has recently been reported to be overexpressed in brain tumors by Cancer Microarray Databases. In this study, we analyzed its expression and role in gliomas.

Materials and Methods: Astrocytic tumor and normal brain tissue samples were obtained from Juntendo and Kanazawa University Hospitals (Japan). Immunoblotting, immunohistochemistry and confocal microscopy were used to visualize the protein expression. Solid-phase binding and pull-down assays were performed to assess the protein-protein interaction. We developed a new endothelial cell-pericyte co-culture assay to mimic the aberrant vasculature *in vitro*.

Results: Tissue immunoblotting analyses showed that Fbln7 is expressed in gliomas and appeared to be overproduced in a grade-dependent manner. Quantification analysis demonstrated the highest expression in GBM compared with the control normal brain or the lower-grade astrocytomas. Immunohistochemically, Fbln7 was highly expressed by endothelial cells and pericytes in the aberrant vessels besides a lower expression by glioblastoma cells. Fbln7 specifically bound to Ang1, but not Ang2 or Tie2, and inhibited the Ang1-Tie2 signal in cultured endothelial cells. In an endothelial cell-pericyte co-culture system, high concentration of VEGF up-regulated Fbln7 expression and resulted in formation of aberrant vessel-like structures, which were suppressed by treatment with neutralizing anti-Fbln7 antibody, Fbln7 peptides or siRNA-mediated down-regulation of Fbln7.

Discussion: We showed the overexpression of Fbln7 in the endothelial cells and pericytes of the aberrant microvascular vessels in GBM. Our study suggests that Fbln7 overproduced by these vascular cells is involved in the formation of dysmorphic blood vessels in GBM through modulation of the Ang1/Ang2-Tie2 signaling pathways by interacting with Ang1. GBM is characterized by an abnormal vasculature, which is one of the molecular targeting therapies. Our data suggest that suppression of Fbln7 function by antibodies or peptibodies or down-regulation of Fbln7 expression may be an attractive target for therapies of GBM when combined with other anti-angiogenic drugs.

Proteomic screening identifies the zonula occludens protein ZO-1 as a new partner for ADAM12 in invadopodia-like structures

B. Dekky; M. Ruff; D. Bonnier; V. Legagneux; N. Théret

Univ Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail)-UMR_S1085, F-35000 Rennes, France

Introduction: The epithelial-mesenchymal transition (EMT) is a key process for cancer cell invasion and migration. This complex program whereby epithelial tumor cells loose polarity and acquire mesenchymal phenotype is driven by the regulation of cell-cell adhesion and cell-substrate interactions. Recently, we demonstrated that the long form of ADAM12 protein promotes EMT. In the present study, we identify ZO-1 as a new partner for ADAM12 during this process and we investigate the role of this complex in invasiveness.
Materials and Methods: To identify interacting partners for ADAM12, we used immunoprecipitation and proteomic approaches on ADAM12L-overexpressed MCF10A cells. By in silico screening, we searched for breast cell line that expressed endogenous ADAM12 and ZO-1. We validated the interaction by immunoprecipitation, by proximity ligation assay and by immunolocalization. By siRNA of ADAM12 or ZO-1 we demonstrated their role in matrix degradation and invasion.
Results & Discussions: A proteomic approach allowed to identify ZO-1 as new partner of ADAM12 during EMT. We showed that ZO-1 and ADAM12 were co-expressed in invasive breast cancer cell lines sharing EMT gene signatures. We validated the interaction between ZO-1 and ADAM12L in breast cancer invasive cell lines where they colocalized in invadopodia-like structures together with membrane type 1 matrix metalloprotease (MT1-MMP) whose activity is required for matrix degradation. In addition, silencing ADAM12L or ZO-1 expression inhibits the activity of matrix degradation and the invasiveness of these cells. Interestingly, we observed that silencing ADAM12L disrupts the localization of ZO-1 at the level of invadopodia-like structures demonstrating the role of ADAM12L in the translocation of ZO-1 to these structures. This distribution of ADAM12 and ZO-1 in the invadopodia type structures is dependent on PKCε protein whose invalidation blocks not only the localization of these proteins but also the activity of matrix degradation and invasion. Together, our data provide evidence for a new interaction between ADAM12, a mesenchymal marker and ZO-1, a scaffolding protein expressed in tight junctions of epithelial cells, both proteins being redistributed at the invadopodia-like structures to promote PKCε-dependent matrix degradation.

Hyaluronan synthesis in fibroblasts is induced by a novel cancer secreted factor

B. Bartolini*; M. L. D'Angelo*; F. Contino†; P. Cancemi†; I. Caon*; E. Caravà*; A. Passi*; D. Vigetti*; E. Karousou*; M. Viola*

Department of Medicine and Surgery, University of Insubria, Varese, Italy; †Centro di Oncobiologia Sperimentale, Oncology Department, La Maddalena, Palermo, Italy

Introduction : Interaction between cancer cells and their microenvironment is central in defining the fate of cancer development. Tumour cells secrete specific signals that modify the surrounding area and the niche supplies structures and activity necessary to survival, growth and development of the cancer cells. In particular, extracellular matrix molecules, like the glycosaminoglycan hyaluronan (HA), are known to influence tumour cell functions, such as proliferation, migration and neoangiogenesis. Here, we show that a yet uncharacterized soluble factor, named c10orf118, secreted by breast tumour cell lines, can induce the secretion of HA by stromal fibroblasts through the up-regulation of the hyaluronan synthase 2 gene (*HAS2*).
Materials and Methods: Conditioned medium from 9701-BC cells was loaded on SDS-PAGE and the representative proteins analysed by MALDI-TOF. The protein expression was investigated in other breast tumour cell lines (MCF-7 and MDA-MB231) by RT-PCR analysis. Co-culture of MCF-7 and normal human dermal fibroblasts was performed to check the rate of expression of *HAS2* and the secretion of HA by fibroblasts.
Results: MALDI-TOF analysis of a band in SDS-PAGE from conditioned medium by 8701-BC cells showed peptides that matched for the c10orf118 protein with the accession number NM_018017 in NCBI/BLAST, corresponding to Q7z3E2 according to the Uniprot identifier. This protein is also expressed by MCF-7 and MDA-MB231 cells, both at intracellular level and secreted in the medium. When co-cultured with MCF-7, fibroblasts showed enhanced expression of *HAS2* and a corresponding increment in HA secretion. These effects could be abolished when the MCF-7 conditioned medium was pre-incubated with an anti-c10orf118 antibody.
Discussion: In this study, we have identified a novel soluble agent that modulates the HA secretion by stromal cells and therefore takes part in tumour matrix remodelling
The expression of the c10orf118 protein associates in cancer patient specimens with the presence of estrogen receptor α, whose signaling pathway has been shown to be influenced by *HAS2* overexpression. We suggest c10orf118 as a new player in regulating the microenvironment remodeling of breast cancer cells, possibly influencing the aggressiveness of the cancer.

LaNt α31, a novel regulator of basement membrane assembly

L. D. Troughton; V. Iorio; V. Barrera; K. J. Hamill

University of Liverpool

Introduction: The laminin N-terminus (LaNt) proteins are a family of laminin- and netrin-related proteins that are derived by alternative splicing from laminin encoding genes. To date, there is only one report of LaNt function, with one family

member, LaNt α31, shown to regulate keratinocyte migration and adhesion through an as yet uncharacterised mechanism. Based on protein architecture, we predicted that LaNt α31 influences laminin organisation.

Materials and Methods: Adenoviral-mediated overexpression of LaNt α31 with C-terminal GFP tag was induced in corneal and epidermal keratinocytes. The impact on cell behaviours was assessed using live cell imaging. LaNt α31 protein interactions and their influence on matrix assembly were determined using immunoprecipitation, indirect immunofluorescence, total internal reflection and live confocal microscopy.

Results: LaNt α31 co-distributed and co-immunoprecipitated with laminin β3 in the extracellular matrix, while live cell assays revealed that the proteins are deposited together during new matrix synthesis. Moreover, induced expression of LaNt α31 led to changes in laminin α3 organisation, forming tight clusters in contrast to the typical broad arcs in control cells. These changes were associated with premature maturation of hemidesmosomes and mis-localisation of focal adhesion complexes. Epithelial cells expressing LaNt α31 GFP also displayed decreased scratch closure and single cell motility rates, and increased cell spreading. All of these aspects could be rescued through provision of a pre-formed matrix indicating a matrix deposition affect rather than cellular defect.

Discussion: Together, these data identify a new protein that can influence the early stages of laminin matrix assembly, introducing the LaNts as new players in regulating cell adhesion and migration.

Dermal collagen XII has a dose-dependent effect on skin repair

K. Schönborn*; S. Willenborg†; J.-N. Schulz†; T. Imhoff‡; A. Niehoff§; S. Eming†; M. Koch‡; T. Krieg*; B. Eckes*

*Translational Matrix Biology, University of Cologne, Germany; †Dermatology, University of Cologne, Germany; ‡Experimental Dentistry and Oral Musculoskeletal Biology, University of Cologne, Germany; §Biomechanics and Orthopedics, German Sport University Cologne, Germany

Introduction: The FACIT collagen XII is expressed in stiff connective tissues where it decorates larger collagen fibrils. Mutations or loss of collagen XII causes disorders of connective tissues in humans, for example, hyperelastic skin, indicating a vital role of collagen XII in development and ECM homeostasis. We postulate that collagen XII has the potential to influence tissue properties by interconnecting structural molecules, transmitting matrix-derived signals to cells or by modulating bioavailability of growth factors.

Materials and Methods: We aim to study the roles of dermal collagen XII during reconstitution of the dermis following excisional wounding in wild type and in mouse lines with fibroblast-specific overexpression or systemic deletion of collagen XII.

Results: In wild-type mice, we observed an increased deposition of collagen XII into the early granulation tissue after injury that persisted at high levels throughout the healing process. The knockout and the overexpressing mouse models both showed delayed wound healing, but a different cellular composition in the granulation tissue. Loss of collagen XII caused wider wounds with elevated numbers of macrophages and myofibroblasts and increased TGFβ signalling. By contrast, overabundance of collagen XII led to thicker wounds with a higher overall cell density and macrophage numbers but without impact on myofibroblasts. As both conditions caused increased macrophage numbers we further characterized the interaction between macrophages and collagen XII *in vitro* and found that only M1 polarized macrophages were able to adhere to collagen XII, but not unstimulated and M2 polarized macrophages.

Discussion: These findings led us to the hypothesis that lack of collagen XII causes a paucity of M1 polarized macrophages and a relative increase in M2 polarized pro-fibrotic macrophages that secrete TGFβ and stimulate myofibroblast differentiation. On the other hand, the overproduction of collagen XII favours a selective adhesion of M1 polarized pro-inflammatory macrophages. To test our hypothesis that collagen XII can control the healing process and possibly its outcome by regulating macrophage populations we will characterize macrophages within the granulation tissue of the two mouse models in detail and investigate the receptor reservoir that mediates the selective adhesion of M1 polarized macrophages to collagen XII.

Wisteria floribunda agglutinin, a perineuronal net marker, demonstrates differential binding affinities to various chondroitin sulphates

A. Goodenough*; W. B. Turnbull*,†; R. P. Richter*,†,‡; J. C. F. Kwok*,§

*School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, United Kingdom; †Astbury Centre for Structural Molecular Biology, University of Leeds, United Kingdom; ‡School of Physics and Astronomy, Faculty of Mathematics and Physical Sciences, University of Leeds, United Kingdom; §Centre for Reconstructive Neurosciences, Institute of Experimental Medicine, Czech Academy of Science, Czech

Introduction: Perineuronal nets (PNNs) are a condensed form of extracellular matrix that encase neurons in the central nervous system and are implicated in a number of disease pathologies and potential treatments, including schizophrenia, epilepsy, autism, spinal cord injury, Alzheimer's disease, addiction and stroke. PNNs contain chondroitin sulphate glycosaminoglycans (CS-GAGs) of which there are 5 subtypes: CS-A to CS-E.

Wisteria floribunda agglutinin (WFA) is a plant lectin which is considered the main marker of PNNs and binds to CS-GAGs in the nets. It is currently thought to specifically bind the GalNAc moiety of the CS-GAGs; if this is the case, then WFA should bind all PNNs. However, evidence has shown that not all PNNs are bound by WFA; thus, we hypothesised that WFA does not simply bind GalNAc but may be specific to certain subtypes of CS-GAG.

Materials and Methods: Quartz crystal microbalance with dissipation monitoring was used to interrogate the PNN/CS interaction. CS-GAG chains of each subtype were secured to an oscillating surface. Mass adsorbed to the surface, in the form of molecules binding, could be detected via a decrease in the frequency of its oscillation.

Results: After addition of WFA at 100 μg/mL to CS-B (DS), CS-D, and CS-E, there were frequency shifts of -8 Hz, -5 Hz and -49 Hz respectively. This suggests that WFA binds CS-E with the highest affinity and can also bind CS-B (DS) and CS-D with lower affinity. CS-A and CS-C showed no frequency shift above that of the control (control: no CS chains bound to surface) indicating that WFA does not bind CS-A and CS-C.

Discussion: The results indicated that WFA is more specific than previously reported, which should be taken into consideration when it is used as a PNN marker. Use of an additional PNN marker such as aggrecan may be advised.

Molecular basis of Asporin as a genetic risk factor for intervertebral disc degeneration

K. S. Lee*; M. S. Yip*; T. K. Lam*; Y. Q. Song*; K. S. E. Cheah*; K. M. C. Cheung†; D. Chan*

**School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China; †Department of Orthopaedics and Traumatology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China*

Introduction: Intervertebral disc degeneration (IDD) is a major cause of back pain, affecting quality of life as we age. While the environment, lifestyle and aging contribute; genetic has a major effect on susceptibility, onset and severity. Previously, we identified Asporin (ASPN), an extracellular matrix (ECM) protein in the intervertebral disc as a risk factor. In addition, increased *ASPN* expression is associated with IDD. *ASPN* appears to have roles in the induction and progression of IDD, but the molecular and degenerative mechanism is not understood.

Materials and Methods: ASPN-tg mice were generated, over-expressing human ASPN under the control of the mouse *Col9a2* promoter, allowing expression in the cartilage endplate and nucleus pulposus (NP) of the intervertebral disc (IVD), leading to changes similar to human IDD. Molecular and cellular changes in the IVD were analysed to gain functional insights. We analysed cell morphology and gene markers for chondrocyte and NP cell maintenance, and performed a proteomic analysis of the NP tissue to assess global and specific changes.

Results: We identified enhanced TGF-β signalling in the NP of ASPN-tg mice, resulting in accelerated chondrogenic events. Interestingly, the most significant change in intervertebral disc (IVD) proteome of ASPN-tg mice is a reduced detectable level of Fibrillin-1 (*FBN1*), a microfibril forming ECM protein that limits TGF-β activation by sequestering the large TGF-β latent complex. Further, changing the genetic background, from C57B/6 to LG/J, considered being poor and good "healer" of damaged tissues respectively, can ameliorate the risk imposed by ASPN on IDD.

Discussion: ASPN-tg mice provided novel mechanistic insights into the functional role of ASPN in the maintenance of NP tissue integrity and function; a process likely to be related to a fine-tuning of TGF-β signalling for ECM integrity, such as the level of FBN1. Indeed, genetic mutations in *FBN1* are associated with the Marfan syndrome, resulting in excessive TGF-β signaling. ASPN-tg mice will provide a mean for additional studies to assess TGF-β signalling and potential of therapeutic interventions, and the possibility to perform genetic mapping to identify protective factors for IDD in the LG/J genetic background.

PCSK9 influences the hyaluronan and proteoglycan metabolism in vascular cells

E. Caravà; I. Caon; E. Karousou; P. Moretto; D. Vigetti; A. Passi; M. Viola

Department of Medicine and Surgery, Insubria University of Varese, via Dunant 5, 21100 Varese, Italy

Introduction: Proprotein convertase subtilisin/kexin type 9 (PCSK9) is involved in regulation of plasma low-density lipoprotein cholesterol (LDL-C) levels in blood directly binding LDL receptor (LDLR) and enhancing its lysosomal degradation in hepatic cells. Therefore, PCSK9 is considered as a major drug target in cardiovascular disease, and antagonizing plasma PCSK9 causes remarkable reductions in LDL-C levels. A new line of investigation has emerged including effects of PCSK9 on matrix biosynthesis in vascular cells. Recently, our laboratory addressed the biology of the atherosclerosis outcome by using vascular cells treated with LDL and oxidized LDL. Such process leads to local responses ending in the production of atherosclerotic plaques. In the early stages of atherosclerosis, hyaluronan (HA) production by endothelial and smooth muscle cells

(SMCs) is critical to form the endothelial glycocalyx that protects vascular tissues from inflammatory cells activation and SMCs are essential in neointima formation. The aim of our study was to explore the effect of PCSK9 in the matrix molecules metabolism in endothelial and smooth muscle cells.

Materials and Methods: Endothelial cells (HUVEC) and smooth muscle cells (hAoSMC) were treated with 20 µg/mL of LDL (normal, oxidized or cholesterol-depleted) and/or PCSK9 (80 or 100 ng/mL) for 24 h. Several genes' expression was quantified using real-time PCR.

Results: We demonstrated that inflammatory stimuli are able to increase the synthesis of HAS2 and not HAS3 in vascular cells and modulate the syndecan 1 and 4 expression on endothelial cells. The incubation of HUVEC cells with PCSK9 partially reduced the LDLR and the receptor for transcytosis ALK1, which are the most expressed in HUVEC. More interestingly, the scavenger receptor LOX1 was dramatically reduced. Preliminary data indicate that the treatment with PCSK9 alters HA and CD44 synthesis both in HUVEC and SMC. In order to evaluate the direct effect of cholesterol on the cells, using cholesterol-depleted LDL, we found an alteration of HA synthesis with these cholesterol-free LDL. These data indicate that HA synthesis depends on the cholesterol amount in LDL and therefore inside the cells.

Discussion: These data support the physiological role played by PCSK9 in HA metabolism by influencing cholesterol content in the cells.

Intracellular trafficking of the invasion promoting cell surface proteinase MT1-MMP

V. Gifford; A. Woskowicz; N. Ito; Y. Itoh

The Kennedy Institute of Rheumatology, University of Oxford, OX3 7FY, United Kingdom

Introduction: Membrane type-1 matrix metalloproteinase (MT1-MMP) is a transmembrane proteinase, which has been shown to promote progression of several diseases including rheumatoid arthritis and cancer by enhancing cellular invasion. MT1-MMP promotes cellular invasion by degrading pericellular extracellular matrix (ECM); thus, its localisation to the leading edge of the cell is crucial. However, the mechanism has not been clearly understood. We have found that MT1-MMP cell-surface exposure is achieved by intracellular trafficking of MT1-MMP containing vesicles along microtubules and identified four kinesin motor superfamily proteins (KIFs) involved in this process.

Materials and Methods: HT1080 human fibrosarcoma cells transfected with siRNA targeting KIFs were subjected to functional analysis of MT1-MMP, including gelatin film degradation assay and collagen film degradation assay. Cell surface localisation of MT1-MMP was analysed by confocal and total internal reflection fluorescence (TIRF) microscopies.

Results: Our data show that the knockdown of four KIFs affects MT1-MMP activity on the cell surface: silencing some KIFs decreased MT1-MMP proteinase activity against gelatin and collagen film, while the knockdown of other KIF enhanced MT1-MMP activity. Interestingly, silencing these KIFs does not affect the overall level of MT1-MMP on the cell surface, while it significantly influences MT1-MMP localisation at the substrate-attached sites of the cells.

Discussion: These data suggest that these four KIFs play key roles in regulating MT1-MMP cell-surface localisation. We hypothesize that each of the four KIFs plays different roles in localising MT1-MMP to the substrate-attachment site. Further investigation of MT1-MMP vesicle transport using live cell imaging techniques will allow us to understand dynamics regulation of MT1-MMP localisation to promote cellular invasion.

Contradictory effects of electroporation on human cutaneous cell migration and proliferation in perspective of wound healing

G. Laure; D. Layal; M. Moinecha; R. Marie-Pierre

Institut de Pharmacologie et Biologie Structurale, IPBS, Université de Toulouse, CNRS, UPS, Toulouse, France

Introduction: When the cell is exposed to high external electric field, the plasma membrane becomes permeable to ions, drugs and molecules as large as plasmid DNA, which are otherwise impermeable: this is electroporation phenomenon. A medical application of electroporation is gene electrotransfer, which is highly promising in wound healing by transferring relevant therapeutic plasmid. Interestingly, in this context, some authors demonstrated that electric field applied alone significantly improved wound closure. Besides, the main clinical application of electroporation in medicine is electrochemotherapy. In this case also, clinicians observed aesthetic and functional wound healing of the treated sites. In brief, even if cellular mechanisms are not yet deciphered, there is a strong link between wound healing and electroporation.

Materials and Methods: We determined the effects of these electric field parameters on primary dermal fibroblasts and keratinocytes isolated from human skin biopsy. We also worked, as a control, with a human melanoma cell line A375.

Results: Gene electrotransfer electric parameters (10 pulses lasting 5 ms at 1 Hz with intensity from 50 to 300 V/cm) did not induce any cell modification in terms of proliferation

and migration abilities for the three cutaneous cell types. Contrariwise electrochemotherapy electric parameters (8 pulses lasting 100 μs at 1 Hz with intensity from 200 to 800V/cm) exerted three distinct effects on the cutaneous cell types. While migration of fibroblasts was stimulated, keratinocytes migration was inhibited, probably because of cell fusion. For A375 melanoma cells, no effect was observed in terms of migration ability.

Conclusion: Further studies are needed to deepen the comprehension of underlying cell mechanisms activated by cell exposition to electroporation. A special focus will be held on extracellular matrix production by fibroblasts after electrostimulation.

Discussion: It is known that a cutaneous wound creates a break in the epithelium and its transepithelial potential which generates an endogenous electric field at the wound margins. In healthy context this endogenous electric field stimulate surrounding cell to achieve an optimal healing, but in chronic wounds, this endogenous electric field is lost. Albeit far from this endogenous electric field characteristics, it seems that electric fields used in electroporation present a potential for electro-stimulation of cutaneous cells in a perspective of wound healing.

Heterogeneity of cancer-associated fibroblasts: Metastatic potential and time determine CAF population composition

F. A. Venning*; K. W. Zornhagen*; T. Thurison*; C. D. Madsen†; J. T. Erler*

Biotech Research & Innovation Centre (BRIC), University of Copenhagen, Ole Maaløes Vej 5, 2200 Copenhagen N, Denmark; †Division of Translational Cancer Research, Lund University, Scheelevägen 2, MV404, Lund, Sweden

Introduction: The metastatic process is heavily influenced by the tumour microenvironment. Cancer-associated fibroblasts (CAFs) are a major class of stromal cells with many, sometimes seemingly conflicting, functions reported. Importantly, CAFs are not a homogenous population of cells. Studies have shown the presence of CAF subpopulations with varying expression patterns of markers, and no specific marker has yet been found to identify all fibroblasts within a tumour. Despite this, CAF heterogeneity is often overlooked in functional studies with only one or two markers being used as identifiers. This approach leads to under-sampling of the entire CAF population and important functional information remains unresolved. We aim to understand how CAF marker heterogeneity corresponds to functional differences. Deciphering the pro- and anti-tumorigenic potentials of CAF subpopulations will deepen our understanding of CAF complexity and ultimately help guide targeted therapies to the correct sub-population(s).

Materials and Methods: Orthotopic, syngeneic murine breast cancer tumours of different ages and metastatic potential were analysed through multi-colour flow cytometry. After lineage exclusion of cancer cells and non-fibroblast stromal cells, the CAF-enriched, lineage population was analysed for combinatorial expression of 6 fibroblast markers.

Results: The composition of the CAF subpopulations within aggressively metastatic tumours was substantially altered from nascent to well-established tumours. However, in less aggressive tumours this change was less pronounced and also different from that of aggressive tumours. Additionally, none of the chosen markers were pan-CAF markers, and importantly, even the combination of all 6 markers did not capture all of the lineage-CAF population.

Discussion: We here identify how a set of 6 commonly used fibroblast markers outline distinct sub-populations of CAFs, and that the composition of CAF sub-populations changes as tumours mature, indicating co-evolution of the CAF population with the tumour. Combining all 6 fibroblast markers failed to label all of the lineage CAFs, underscoring the heterogeneity of CAFs, and the need for novel CAF markers.

Collagen XIII contributes to transsynaptic adhesion in the developing neuromuscular junction

N. Oula; H. Anne; P. Taina

Oulu Center for Cell-Matrix Research, Biocenter Oulu, Faculty of Biochemistry and Molecular Medicine, University of Oulu, Finland

Introduction: Collagen XIII is a type II transmembrane protein associated with neuromuscular and myotendinous junctions in the muscle. In the neuromuscular junction, collagen XIII localizes to the synaptic basal lamina and postsynaptic membrane. Loss-of-function mutations in the *COL13A1* gene lead to congenital myasthenic syndrome type 19. Typical symptoms are muscle weakness and fatigability, leading to respiratory and feeding difficulties, and thus, the condition is usually very severe. Collagen XIII has been found necessary for correct morphology, transsynaptic adhesion, alignment, maturation and function of the neuromuscular junction, but little is known about its effects during embryonic neuromuscular development.

Materials and Methods: The diaphragm was collected as a whole-mount preparation during embryonic development from wild-type and collagen XIII-knockout mouse foetuses and stained for neuromuscular junctions and motor neurons. Samples were analysed with confocal microscopy followed by quantification and statistical analyses.

Results: In mouse foetuses lacking collagen XIII, the pattern formed by neuromuscular junctions is significantly wider in the diaphragm muscle at embryonic day 16,5. Also, in the absence of collagen XIII there is an increased number of motor neuron axons that fail to stop at the neuromuscular junctions, growing beyond them instead. Preliminary results indicate that lack of collagen XIII leads to increased branching of the phrenic nerve and especially the number of terminal branches appears larger.

Discussion: These findings suggest that collagen XIII has a significant role in transsynaptic adhesion between muscle and nerve, and in conveying an attachment signal to establish the motor synapse during neuromuscular development. In addition, collagen XIII expression has been detected in vasculature surrounding the phrenic nerve and other peripheral nerves, and the increased branching of the phrenic nerve observed in collagen XIII-knockout mice might suggest a role for collagen XIII in regulating axon outgrowth.

Validation of murine primers for the characterization of heparan sulfate biosynthesis enzymes during physio-pathological processes

Y. Salemkour; A. Pecq; A. Maiza; D. Le Moigne; E. Hay; A. Fifre; D. Papy-Garcia; P. Albanese

ERL CNRS 9215, Laboratoire Croissance, Régénération, Réparation et Régénération Tissulaires (CRRET)/Université Paris Est Créteil, Université Paris Est, 94010, Créteil, France

Introduction: Heparan sulfate (HS) is a family of linear sulfated glycosaminoglycans linked to core protein to form proteoglycans, on cell membrane or in the extracellular matrix. HS are characterized by D-glucuronic acid / N-acetyl-D-glucosamine disaccharides polymerized in chains and modified by several classes of enzymes, like sulfotransferases (OSTs) that add sulfates at various positions of the disaccharides. HS structures and functions are modified during pathophysiological evolution of a tissue, such as aging or injury; then, it is very important to study pattern of expression of all the enzymes involved in HS biosynthesis.

Materials and Methods: We present molecular biology tools and reliable quality criteria, such as primers sequences design and tissue controls validation, for specific RT-qPCR analysis of the expression pattern of 26 enzymes linked to HS modification in murine models. RT-PCR analysis was performed according to Agilent Technologies, to determine Cq, standard and melting curves to validate amplicons purity and Tm dissociation.

Results: All primer pairs must respect several design criteria in order to be specific of the gene of interest. This specificity as well as the amplification efficiency was validated in tissues positive controls identified *in-silico* as putative on https://www.proteinatlas.org/ database. Most of the enzymes are expressed in neural tissues, such as Cortex and Cerebellum, as well as in spleen, rate and chondrocytes, that were used as positive control. Finally, all enzymes expressions were analyzed in 2 models of osteoarthritis, a degenerative pathology of the cartilage characterized by a strong remodelling of the glycanic compounds of the matrix: (1) *in vitro* on primary culture of murine chondrocytes induced toward hypertrophic phenotype by IL-1 and (2) *in vivo* in mice with chirurgical Destabilization Medial Meniscus.

Discussion: The molecular tools developed here permit to complete our glycomic platform: data on structural evolution of GAG that we recently obtained in OA can be now correlated with HS enzymes' expression patterns. This confirms our ability to identify glycanic targets for new therapeutic strategy of degenerative pathologies.

Fibroblast state switching orchestrates dermal maturation and wound healing

E. Rognoni*; A. O. Pisco*; T. Hiratsuka*; K. Sipilä*; J. M. Belmonte†; S. A. Mobasseri*; C. Philippeos*; R. Dilão‡; F. M. Watt*

**Centre for Stem Cells and Regenerative Medicine, King's College London, 28th Floor, Tower Wing, Guy's Hospital Campus, Great Maze Pond, London SE1 9RT; †Developmental Biology Unit and Cell Biology and Biophysics Unit, European Molecular Biology Laboratory, Heidelberg, Germany; ‡Nonlinear Dynamics Group, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal*

Introduction: The maturation of dermis, as well as wound healing, is a tightly controlled combination of fibroblast proliferation, quiescence, and extracellular matrix (ECM) secretion and organization. Here, we explore the transitions in fibroblast behaviour by developing a novel virtual tissue model for recapitulating how fibroblast subpopulations emerge, arrange and influence each other during development and wound healing.

Materials and Methods: The methods combine state-of-the-art mathematical and computational modelling, *in vivo* live imaging, histological analysis (including collagen analysis by picrosirius, collagen hybridizing peptide, and TEM), lineage tracing, as well as *in vitro* experiments (collagen gel and organotypic culture) with isolated human and mouse fibroblasts.

Results: Based on the *in vivo* and *in silico* analysis of fibroblast behaviour, we propose a model where dermal architecture is determined by a negative feedback loop between ECM deposition and proliferation. *In vitro* experiments with primary fibroblasts supported the idea of

ECM-proliferation feedback loop and showed that ECM deposition alone is sufficient to drive neonatal fibroblast to quiescent state. On the contrary, the destruction of collagen mimicking the wound situation activated the proliferation of fibroblasts. Our model describes dermal maturation process and the loss of spatial segregation of fibroblast lineages. In addition, the model suggests that migration of fibroblasts is needed for wound healing but not for developmental maturation leading to the homeostatic skin architecture. These predictions were confirmed by *in vivo* analysis.

Discussion: Our results provide a mechanistic insight how the fibroblast behaviour is coordinated to reach the normal maturation of dermis and wound healing. This will be useful for understanding the pathological processes of skin and for identifying new potential therapies.

Endothelial cell-derived MMP-14 is dispensable for skin formation and repair

S. Neumann; M. Kümper; C. Mauch; P. Zigrino

Department of Dermatology, University of Cologne, Cologne, Germany

Introduction: Angiogenesis, the formation of new blood vessels from pre-existing ones, is a natural and crucial process during development and in adulthood. MMP-14, a zinc-dependent endopeptidase, plays a critical role in angiogenic processes. MMP-14 deficiency is lethal within 3 weeks, and mice display severe skeletal defects.

Materials and Methods: To investigate the cell-autonomous role of MMP-14 expression in endothelial cells, we generated a conditional knockout mouse line with endothelial cell-specific depletion (Tie2-driven Cre) of MMP-14 (MMP-14 $EC^{-/-}$). These mice are mostly normal and have a normal lifespan.

Results: Similar to the complete MMP-14 knockout, the skull of MMP-14 $EC^{-/-}$ mice at postnatal day 7 displayed slightly delayed suture closure, a slightly domed skull, and a shortened snout previously accredited to the defective osteoblast-MMP-14 activities. Strikingly our data demonstrate that this phenotype can result from ablation of MMP-14 in endothelial cells, revealing a crucial role for endothelial MMP-14 in membranous ossification and suture closure. Adult mice (3 months) also developed shortened snouts with deviations of the midface, whereas long bones (femurs) were completely normal.

Histological skin analysis revealed overall normal skin morphology and epidermal differentiation patterns. Also wound repair in MMP-14 $EC^{-/-}$, including kinetics, re-epithelialization, epidermal differentiation and granulation tissue formation were comparable with wild-type mice. Further, no altered neovascularization at day 5 and day 17 was shown (CD31/SMA stainings), and collagen density and fibers at later time point were comparable to control mice. To investigate whether single MMP-14-deficient endothelial cells showed molecular and functional defects when grown in a simplified dermis-like culture system in vitro, we embedded isolated endothelial cells as single cells in a three-dimensional fibrillar collagen matrix and induced the formation of a vascular network with various stimuli. In this culture system, isolated MMP-14 $EC^{-/-}$ endothelial cells formed some tube-like structures similar to controls, but did not penetrate the gels. In these cultures, in the absence of MMP-14, we detected large areas of clustered and flattened cells.

Discussion: Taken together, these studies showed that in vivo deletion of MMP-14 in endothelial cells, whereas important for membranous ossification and suture closure in skulls development, is dispensable during skin developmental processes and homeostasis.

Laminin-511 controls melanocyte differentiation by regulating their migration

Y. Üstün[*,†]; M. Reibetanz[†]; B. Brachvogel[‡]; Z.-L. Chen[§]; B. Eckes[*,†]; P. Zigrino[†]; R. Nischt[†]; T. Krieg[*,†]

[*]Translational Matrix Biology, University of Cologne, Germany; [†]Dermatology, University of Cologne, Germany; [‡]Experimental Neonatology, University of Cologne, Germany; [§]Laboratory of Neurobiology and Genetics, The Rockefeller University, New York, USA

Introduction: Laminins, the major basement membrane (BM) components, are heterotrimeric glycoproteins, composed of α, β and γ chains. In skin, the main laminin isoforms in the dermo-epidermal BM are laminin-332, laminin-511 and laminin-211, the latter being restricted to hair follicles (HFs). Laminin γ1 chain is the most abundant γ chain, deletion of which leads to early embryonic lethality at day E5.5.

Materials and Methods: To elucidate the cellular function of γ1 chain in skin, we generated mice with keratinocyte-specific deletion of this chain ($Lamc1^{EKO}$) by using the keratin 14-Cre/*loxP* system.

Results: Strikingly, $Lamc1^{EKO}$ mice show delayed and reduced coat pigmentation. This was not due to a reduction in total melanocyte numbers, but attributable to reduced expression of differentiation specific enzymes TRP-1, TRP-2 and tyrosinase by melanocytes. Since melanocytes in controls differentiate upon migrating into HFs, we postulate that in $Lamc1^{EKO}$ skin, the migratory capacity of melanocytes into HFs may be impaired, resulting

in an abnormal distribution within the skin. Indeed, melanocytes in skin of *Lamc1*^{EKO} mice were retained in the epidermis and were less abundant in HFs. The observed defective melanocyte migration was explained by the loss of keratinocyte-derived laminin-511 and ectopic deposition of fibroblast-derived laminin-211 in the whole dermo-epidermal BM of *Lamc1*^{EKO} mice. This is concordant with our finding that laminin-511, but not laminin-211, is the preferred substrate for migration and adhesion of primary melanocytes *in vitro*.

Besides an appropriate extracellular matrix supporting migration, melanocyte migration into HFs also depends on the chemoattractant SDF1 (Stromal cell-derived factor 1), which acts through its receptor CXCR4 being expressed on melanocytes. Interestingly, laminin-511, but not laminin-211, induces the expression of CXCR4 on melanocytes, required for SDF1-mediated migration. By contrast, total expression levels of SDF1 in skin of *Lamc1*^{EKO} mice did not differ from controls.

Discussion: In summary, we show that laminin-511 regulates the differentiation of melanocytes by two independent mechanisms that control their migration from the epidermis into HFs. First, laminin-511 serves as migration substrate. Second, it stimulates the expression of CXCR4 on melanocytes and their recruitment into HFs in an SDF1-dependent manner.

The gC1q domain is responsible for EMILIN1-induced lymphangiogenesis

A. Capuano; E. Pivetta; F. Baldissera; G. Bosisio; F. Bucciotti; R. Doliana; P. Spessotto

Molecular Oncology, Department of Translational Research, CRO-IRCCS, Aviano, Italy

Introduction: Lymphatic vessels (LVs) play a pivotal role in the control of tissue homeostasis and have also emerged as important regulators of immunity, inflammation and tumor metastasis. EMILIN1 is the first ECM protein identified as a structural modulator of the growth and maintenance of LV; accordingly, *Emilin1*$^{-/-}$ mice display lymphatic morphological alterations leading to functional defects (mild lymphedema, leakage and compromised lymph drainage). Many EMILIN1 functions are regulated by the ligand-receptor interaction of its gC1q domain: the E933 within a protruding loop of the gC1q structure is the site of interaction with α4/α9β1 integrins and, in contrast to a large body of evidence that signals generated by ligand-activated integrins are pro-proliferative, gC1q binding reduces cell proliferation.

Materials and Methods: We produced E933A EMILIN1 transgenic mice (E933A-TG), expressing a mutant EMILIN1 unable to be engaged by integrin receptors. Morphological analyses and lymphangiography assays were then performed to investigate the specific regulatory control of the gC1q domain on lymphangiogenesis, together with in vitro tubulogenesis tests.

Results: E933A-TG presented abnormal architecture of LVs with dense, tortuous and irregular networks; moreover, the number of anchoring filaments was reduced and collector valves displayed aberrant narrowed structures. E933A mutation affects also lymphatic function in lymphangiography assays and makes TG mice more prone to develop LN metastasis. The hypothesis that gC1q is the most important domain to induce a correct lymphangiogenesis response was confirmed and reinforced by functional *in vitro* tubulogenesis tests (in which EMILIN1, gC1q WT but not E933A mutant recombinant stimuli were able to organize LECs in a 3D-network of tubes). In addition, *ex vivo* thoracic-duct ring assay revealed that TG-derived LECs show severely reduced sprouting capacity and are not able to organize into capillary-like structures.

Discussion: All both morphological and functional evidences, describing for E933A-TG mice a lymphatic phenotype very similar to that already observed for the *Emilin1*$^{-/-}$ background, clearly indicate that the most important EMILIN1 element able to induce a correct lymphangiogenesis response is within the C-terminal domain gC1q and that it involves the interaction with the integrin.

Lymphoid-like extracellular matrix partially polarizes stroma cells toward a lymphoid phenotype

L. Verdière; D. Roulois; V. Sibut; F. Mourcin; K. Tarte

UMR_S 1236, Université Rennes 1, INSERM, Etablissement Français du Sang, Labex IGO, F-35000, Rennes, France

Introduction: Lymph nodes are organized into different niches to support efficient encounter between lymphocytes and antigens. Those niches are structured by lymphoid stromal cells (LSC) that synthesise specific extracellular matrices (ECM). It has been shown that LSC modulate B cells migration, survival and antigen encounter. However, little is known on the role of ECM in lymph nodes. This project aim was to assess the effect of ECM on the polarization of stromal cells.

Materials and Methods: Tonsil stromal cells (TSC) and adipose-derived stromal cells (ADSC) were cultured during ten days with or without TNFa and LTa1b2 to induce a LSC phenotype. Cells were then decellularized to remove cells but not the ECM. TSC and ADSC were then cultured for three days on ECMs and lymphoid stromal cell markers (ICAM-1,

VCAM-1 and GP38) expressions were evaluated by flow cytometry.

Results: Flow cytometry assays show an increase expression of ICAM-1 on both stromal cells types (TSC and ADSC) only when they are cultivated on lymphoid-like ECMs produced by ADSC polarized into LSCs. In contrast, VCAM-1 and GP38 expressions do not change.

Discussion: These first results show that lymphoid-like ECM can modify stromal cell expression for lymphoid stromal cell marker. This suggests a mechanism of LSC self-induction in lymphoid organ, with ECM microenvironment encouraging stromal cell polarization to LSC phenotype. Despite those encouraging results, the ECM effect on stromal cell polarization remains unclear and further analyses are required to better understand this phenomenon. Tests on B cells are currently being made to assess ECM influence on their survival and proliferation. Moreover, RNAseq analysis on different stromal subsets in human is ongoing. It will help us to identify specific ECM molecules that could play a role in the adaptive immune response.

Regulation of Metalloproteinases during Ovulation in Zebrafish

Y. Zhu*,†; D. T. Liu*,†; N. J. Carter*; X. J. Wu*; S. X. Chen†; W. S. Hong†

Department of Biology, East Carolina University, 1000 5th Street, Greenville, North Carolina 27858, United States of America; †State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, Fujian Province 361102, People's Republic of China

Introduction: Ovulation requires proteinases to promote the rupture of ovarian follicles. However, the identity of these proteinases remains unclear. In our previous studies using RNA-seq analysis of differential expressed genes, we found significant down-regulation of five metalloproteinases: *adam8b* (a disintegrin and metalloproteinase domain 8b), *adamts8a* (a disintegrin and metalloproteinase with thrombospondin motif 8a), *adamts9*, *mmp2* (matrix metallopeptidase 2), and *mmp9* in the nuclear progestin receptor knockout ($pgr^{-/-}$) zebrafish that have failed to ovulate. We hypothesize that these metalloproteinases are responsible for ovulation and are regulated by progestin.

Materials and Methods: In this study, we first determined the expression of these five metalloproteinases and *adamts1* in preovulatory follicles at different times within the spawning cycle in $pgr^{-/-}$ and wild-type (*wt*) zebrafish and under varying hormonal treatments.

Results: We found that transcripts of *adam8b*, *adamts1*, *adamts9* and *mmp9* increased drastically in the preovulatory follicular cells of *wt* female zebrafish, while changes of *adamts8a* and *mmp2* were not significant. This increase of *adam8b*, *adamts9* and *mmp9* was significantly reduced in $pgr^{-/-}$, whereas expression of *adamts1* was not affected in $pgr^{-/-}$ zebrafish. Interestingly, expression of two key upstream regulators, *pgr* and the luteinizing hormone/choriogonadotropin receptor (*lhcgr*), increased sequentially in preovulatory follicular cells in *wt* fish prior to the increase of metalloproteinases *in vivo*. Thereafter, we focused on hormonal regulation of the *adamts9* proteinase because of its specific expression in the follicular cells. Strong immunostaining of Adamts9 was observed in the follicular cells of *wt* fish, and this expression was reduced drastically in $pgr^{-/-}$. Importantly, preovulatory follicles markedly increased *adamts9* expression in a dose, time and Pgr-dependent manner when stimulated by 17α,20β−dihydroxy-4-pregnen-3-one (DHP, a progestin) *in vitro*. Exposure to human chorionic gonadotropin (hCG) alone had no effect on expression of *adamts9* or oocyte maturation. Upon steroid (DHP, RU486, or testosterone) induced oocyte maturation, hCG could further stimulate *adamts9* expression both in *wt* and $pgr^{-/-}$zebrafish.

Discussion: Our results provide the first evidence that hormonal upregulation of *adamts9* occurs specifically in preovulatory follicular cells of zebrafish prior to ovulation. *Adamts9* is primarily regulated by progestin and then reinforced by gonadotropin via their cognate receptors.

Ubiquitous removal of ccn2 in a bleomycin induced pulmonary fibrosis model is detrimental to survival

A. L. Horwell*; K. Liu*; L. Spencer†; R. Moots†; R. Van 'T Hof*; G. Bou-Gharios*

Institute of Ageing and Chronic Disease, Faculty of Health & Life Sciences, University of Liverpool; †Department of Rheumatology, Clinical Sciences building, University Hospital Aintree, Liverpool

Introduction: Idiopathic pulmonary fibrosis (IPF) is a chronic progressive form of lung fibrosis that is ultimately fatal in all cases. Mortality rates at 5 years after diagnosis of IPF are only 20%. Current treatment options fail to prevent progression or cure this disease. Fibrotic lung disease is characterised by over-production and deposition of collagen I; however, the mechanism behind the initiation and propagation of this process is unknown.

CCN2 has been shown to be upregulated in patients with IPF and in animal models of pulmonary fibrosis. We tested whether the ablation of CCN2 from fibroblasts or all cells would ameliorate the pathology.

Materials and Methods: We mated a floxed CCN2 mouse line with either of two inducible CreERT2 recombinase transgenic mouse lines to examine the effects of removing CCN2 in a fibroblast specific (Col1a2-cre) and ubiquitous (ROSA26-cre) manner, followed by a bleomycin-induced

model of IPF. Bleomycin model was carried out via oropharyngeal aspiration route with a single dose of 0.375 ng/g bleomycin administered in sterile PBS. Lungs were compared with untreated mice using a Bruker Skyscan 1272 ex-vivo μCT scanner to identify regions of tissue deposition and compare air with tissue densities. A single lobe from each lung was analysed histologically to confirm the fibrotic pathology and tissue composition. RNA was extracted from the remaining lobes for qPCR analysis. Changes in gene expression were determined using a ΔΔCT calculation.

Results and Discussion: The data showed that the removal of CCN2 from fibroblast cells did not provide protective effect at day 14 post–bleomycin-induced pulmonary fibrosis. However, when CCN2 was removed ubiquitously it resulted in a severe fibrosis with an accelerated rate of matrix synthesis and deposition. This suggested that in vivo, CCN2 plays a regulatory role to limit the rate of matrix production, rather than being the culprit molecule as suggested in the literature. Therefore, we conclude that any attempts to block CCN2 universally would be detrimental to lung architecture and function.

Inhibition of heparanase protects against renal failure and fibrosis following ischemia/reperfusion

V. Masola*,†; G. Bellin*,†; G. Vischini‡; L. Dall'Olmo§; G. Gambaro‡; A. Lupo*; M. Onisto§

*Renal Unit, Department of Medicine, University-Hospital of Verona, Verona, Italy; †University of Padova, Department of Biomedical Sciences Padova, Padova, Italy; ‡Università Cattolica del Sacro Cuore, Roma, Italia; §Azienda Ulss 3 Serenissima - Ospedale San Giovanni e Paolo - Venice, Italy

Introduction: Renal ischemia/reperfusion (I/R) injury occurs in patients undergoing renal transplantation and with acute kidney injury (AKI). It is responsible for the development of chronic allograft dysfunction characterized by parenchymal alteration and fibrosis. Heparanase (HPSE), an endoglycosidase that regulates EMT and macrophage polarization, is an active player in the biological response triggered by ischemia/reperfusion (I/R) injury.

Materials and Methods: I/R was induced in vivo by clamping left renal artery for 30 min in wt C57BL/6J mice. Animals were daily treated or not with roneparstat (an inhibitor of HPSE) and sacrificed after 8 weeks. HPSE, fibrosis, EMT-markers, inflammation and oxidative stress were evaluated by biomolecular and histological methodologies together with the evaluation of renal histology and measurement of plasmatic and urine parameters of renal function.

Results: Eight weeks after I/R, HPSE was upregulated in both renal parenchyma and plasma whereas tissue specimens showed clear evidences of renal injury and fibrosis (IF/TA). The inhibition of HPSE with roneparstat restored histology and fibrosis level comparable with that of control. I/R injured mice showed a significant increase of the EMT, inflammation and oxidative stress markers, but they were significantly reduced by the treatment with roneparstat. Finally, the inhibition of HPSE in vivo almost restored renal function as measured by BUN, plasma creatinine and albuminuria.

Discussion: The present study points out that HPSE is actively involved in the mechanisms that supervise the development of renal fibrosis arising as a consequence of the ischemia-reperfusion damage. In the transplanted organ, HPSE inhibition would therefore constitute a new pharmacological strategy to reduce.

Extracellular matrix induced by aldosterone through a G-protein-coupled receptor revealed in a novel Drosophila model of renal fibrosis

M. Tatar*; W. Zhang*; K. Ocorr†

Division of Biology and Medicine, Brown University, Providence, Rhode Island, USA; †Neuroscience and Aging Research Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, USA

Introduction: Extracellular matrix accumulates in the course of renal fibrosis. One potential entry to modulate this pathology is through control of steroid hormones. Notably human aldosterone appears to foster fibrosis when it is chronically expressed, as in diabetes. Aldosterone may affect fibrosis through the mineralocorticoid nuclear hormone receptor, yet some reports implicate an unidentified, non-canonical receptor. With a novel Drosophila model system of fibrotic disease and ECM accumulation, we describe such an alternative in the form of a G-protein-coupled receptor, Drosophila dopEcR.

Materials and Methods: To quantify renal fibrotic disease in Drosophila we measured protein secretion in urine (proteinuria) and impaired nephrocyte filtration (glomerular disease). Extra-cellular matrix pericardin (collagen IV) was quantified as mRNA from excised nephrocyte/cardia tissue, by immunostaining, and by Western analysis. We fed human aldosterone and Drosophila steroid hormones (ecdysone and 20-hydroxyecdysone) to adults for 3 w to test their chronic impact on fibrosis. With tissue specific drivers, we expressed RNAi against candidate genes to identify the receptor and tissue responsible for steroid-induced fibrosis.

Results: Aldosterone and ecdysone strongly induce fibrosis in the Drosophila renal system, measured as proteinuria, impaired nephrocyte filtration, and accumulation of ECM

collagen (pericardin). All pathology arose from pericardin produced by cardia-myocytes. Steroid induce fibrosis was not ameliorated by RNAi against the Drosophila canonical nuclear hormone receptor EcR, but all were blocked by knockdown of dopEcR, a membrane-associated G-protein-coupled receptor.

Discussion: Human aldosterone and Drosophila ecdysone signal through a novel GPCR to modulate ECM accumulation and fibrotic pathology of the renal system. The GPCR dopEcR is a dual ligand receptor responsive to dopamine and ecdysone, previously described to affect select neuronal behaviours. We find it also acts in heart muscle cells, perhaps reflecting myofibroblast-like function, to structure expression of pericardin (collagen IV) in the cardia-renal tissue. Chronic exposure to aldosterone or ecdysone stimulates excess ECM and disrupts both renal and heart function. Potential homologs of dopEcR occur in humans, and may present targets for pharmacological approaches to repress fibrotic disease.

The importance of tissue transglutaminase for the deposition of matrix proteins in idiopathic pulmonary fibrosis

S. Fell*; C. B. Nanthakumar†; M. Griffin*

*School of Life and Health Sciences, Aston University, Birmingham, United Kingdom; †Fibrosis DPU, Respiratory TA, GlaxoSmithKline, Stevenage, United Kingdom

Introduction: Idiopathic pulmonary fibrosis (IPF) is a progressive and life-threatening disease that has 5000 new cases diagnosed each year in the UK with a median survival of less than 3 years. Previous studies show tissue transglutaminase (TG2) expression and activity is elevated in the lungs of IPF patients; however, the role of TG2 in IPF has not yet been elucidated. TG2 is a multifunctional enzyme that has been implicated in various pathological conditions. This study aims to validate TG2 as a therapeutic target for IPF.

Materials and Methods: Primary Normal Human Lung Fibroblast (NHLF) and fibroblasts derived from patients with IPF were cultured in DMEM supplemented with 10% FCS and 1% NEAA. NHLF cells were transduced using a WT-TG2 or catalytically inactive TG2 (C277S) carrying lentivirus. Protein levels in whole-cell lysates and the ECM were measured via Western blotting. Extracellular fibronectin and collagen was measured using immunocytochemistry and high-content imaging. TGFβ activity levels were measured using a Mink Lung Epithelial Cell (MLEC) TGFβ reporter assay. CRISPR-Cas9 genome editing was performed on IPF fibroblasts targeting the *TGM2* gene.

Results: These data show that stimulation of NHLF with TGFβ1 markedly increases TG2, collagen and fibronectin deposition into the matrix. The addition of exogenous TG2 elevates TG2, fibronectin and TGFβ1 deposition into the matrix, whilst also increasing levels of active TGFβ. The lentiviral transduction of WT-TG2 into NHLF cells increases matrix deposition of fibronectin and TGFβ1; however, this is ameliorated in NHLF cells transduced with a C277S-mutant TG2. The application of a cell-impermeable TG2-specific inhibitor prevents TGFβ1-induced increases in TG2 and fibronectin deposition in NHLF cells, whilst also decreasing TG2 and fibronectin matrix deposition in IPF fibroblasts. Finally, CRISPR-Cas9 genome editing targeting *TGM2* in IPF fibroblasts leads to a reduction in collagen deposition.

Discussion: This study demonstrates that TG2 plays a vital role in the deposition of collagen, fibronectin and TGFβ1. Further to this, these data demonstrate that it is extracellular, catalytically active TG2 that is required to elevate matrix deposition of proteins in healthy lung fibroblasts. Most importantly, targeting TG2 in IPF fibroblasts leads to a marked reduction in matrix protein deposition.

Differences between the matrix of the neonatal and adult extrahepatic bile duct: implications for injury

J. Llewellyn*; G. Khandekar*; A. Kriegermeier†; A. Wehrman†; N. D. Theise‡; R. G. Wells*

*Department of Medicine (Gastroenterology), The University of Pennsylvania, Philadelphia, PA, 19104, United States; †Division of Gastroenterology, Hepatology and Nutrition, The Children's Hospital of Philadelphia, Philadelphia, PA, 19104, United States; ‡Department of Pathology, New York University, School of Medicine, New York, NY 10016, USA

Introduction: The submucosal interstitial space of the extrahepatic bile duct (EHBD) is a poorly characterized space that is known to contain organized bands of collagen and fluid flow in adults. Its structure in neonates is not known, but may be important in diseases such as biliary atresia that specifically affect the neonatal EHBD. We carried out detailed analysis of the ECM of the neonatal compared to the adult mouse EHBD in an effort to identify anatomical features that might render neonates particularly susceptible to injury.

Materials and Methods: EHBDs were isolated from adult mice and pups at postnatal days 0-15. These were imaged using transmission electron microscopy, second harmonic generation microscopy and were stained for matrix components and cell type markers.

Results: The submucosal space of EHBDs in adult mice consists of collagen I/III bundles, proteoglycans, hyaluronic acid and elastin, interspaced with fluid-filled spaces. The collagen bundles are lined by fibroblasts. Conversely, the neonatal EHBD interstitium has minimal elastin and collagen; these are progressively deposited in the first 15 days of life. The

diameter of collagen fibrils and expression of fibrillogenesis-associated proteoglycans lumican and fibromodulin change in parallel during postnatal development. Neonatal submucosal cells are more rounded and contain large amounts of rough endoplasmic reticulum, suggesting that they are responsible for collagen deposition. Many of these cells in the neonate stain for myofibroblast marker αSMA and mesenchymal stem cell marker CD105.

Discussion: The extracellular matrix and phenotype of cells present in the submucosal space of the EHBD significantly changes from birth to adulthood. The early presence of space filled primarily with proteoglycans and lacking major structural proteins of the matrix suggests that neonatal EHBDs will have poor response to mechanical stress (eg, obstruction) and that injurious material (eg, bile, toxins, viruses) is more likely to spread within the submucosal interstitium. The presence of a larger αSMA-positive and metabolically active cell population raises the possibility that the neonatal EHBD is primed to respond to injury and fibrotic cues. Future work will focus on characterising the mechanical responsiveness and fluid flow of the neonatal vs. adult duct and on assessing the fibrogenic cell population at different stages of development.

A 3D model of human mature lysyl oxidase

S. D. Vallet*[,1]; M. Guéroult[†,1]; N. Belloy[†,‡]; M. Dauchez[†,‡]; S. Ricard-Blum*

**University Lyon 1, CNRS, Institute of Molecular and Supramolecular Chemistry and Biochemistry, UMR 5246, Villeurbanne cedex, France; [†]UMR URCA/CNRS 7369 Matrice Extracellulaire et Dynamique Cellulaire; [‡]Plateau de Modélisation Moléculaire Multi-échelle, Université de Reims Champagne-Ardenne, France*

Introduction: Lysyl oxidase (LOX, protein-lysine 6-oxidase) catalyses the oxidative deamination of lysyl and hydroxylysyl residues in collagens and elastin, and the first step of their covalent cross-linking. LOX expression is up-regulated in fibrosis and metastasis. No 3D structure of the catalytic domain of LOX is available, preventing the design of specific inhibitors. This prompted us to build a 3D model of human LOX.

Materials and Methods: Robetta was used to build fragment libraries of 3 and 9 residues based on the structures available in the Protein Data Bank, and distant homologs of the human LOX sequence based on hidden Markov models were retrieved with HHpred. 5,000 models were generated by Rosetta using Robetta fragment libraries, selected templates from HHpred, and structural features (copper ion, the lysyl-tyrosylquinone—LTQ—cofactor and disulfide bonds). Long molecular dynamics simulations were run to assess the model stability with Gromacs software and the amber99SB-ildn*-q force field including modified parameters for copper ions.

Results: The model has a V-shaped structure with a groove, which contains the catalytic site in close contact with LTQ, and can accommodate a collagen triple-helix. The groove acts as a hinge axis leading either to an open conformation, where the catalytic site is accessible, or to a closed one where it is buried. This motion is supported by the fact that the radius of gyration of the model is correlated with the hinge angle.

Discussion: This model is the first one to recapitulate all known molecular features of LOX (the copper ion coordinated by three histidine residues, LTQ and five disulfide bridges). It will be useful to perform docking experiments with LOX substrates and other partners to decipher its molecular mechanisms of action, and to design new LOX inhibitors for therapeutic purpose.

[1]Equal contribution.

Scleraxis plays an indispensable contribution to progenitor lineage direction in adult tendon wound healing

T. Sakai

Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool, Liverpool, L69 3GE, United Kingdom

Introduction: Tendon is a dense connective tissue that transmits high mechanical forces from skeletal muscle to bone. Adult tendon injuries occur very frequently, but injured tendon heals very slowly and the mechanisms of the slow-healing response to injury are still largely unknown. A transcription factor Scleraxis (Scx) is a highly specific marker of precursor and mature tenocytes. Mice lacking *scx* show a specific and virtually complete loss of tendons during development. However, the functional contribution of Scx to adult tendon wound healing has not yet been fully characterized.

Materials and Methods: We developed a simple and reproducible Achilles tendon "partial-transection" injury model, and utilized a combination of *ScxGFP*-tracking and loss-of-function systems. Mouse adult tendon progenitor cell lines were generated from adult *scx(flox/flox)/ScxGFP* mouse Achilles tendon under a *Trp53*- and *Cdkn1a* (*p21*)-null genetic background.

Results: We show here that paratenon cells, representing a stem cell antigen-1 (Sca-1)-positive, Scx-negative progenitor subpopulation, display Scx induction, migrate to the wound site and produce extracellular matrix (ECM) to bridge the defect, whereas resident tenocytes exhibit a delayed response. The induction of Scx in the progenitors is initiated by TGF-β-signaling. The *scx*-deficient mice had migration of Sca-1-positive progenitor cell to the lesion site following injury, but impaired ECM assembly to bridge the defect. Mechanistically, scx-null progenitors displayed higher

chondrogenic potential with up-regulation of SRY-box 9 (Sox9) coactivator PPAR-gamma coactivator 1α (PGC-1α) *in vitro*, and knock-in analysis revealed that forced-expression of full-length *scx* significantly inhibited *sox9* expression. Accordingly, *scx*-null wounds formed cartilage-like tissues that developed ectopic ossification.

Discussion: Our comprehensive studies of adult tendon wound provide the following compelling evidence: 1) Scx plays indispensable roles in proper healing following adult tendon injury; 2) there is a direct link between tendon progenitor cell lineage mediated by Scx and adult tendon pathology; and 3) certain Sca-1-positive progenitor subpopulations identified in the paratenon could provide novel targets to develop strategies to facilitate tendon repair. We propose that the regulatory mechanisms underlying lineage-specific differentiation in adult tissue progenitors shown here could be translated in a broader variety of tissues or systems in the body.

Novel non-invasive biomarkers of extracellular matrix remodeling reflect the burden of renal fibrosis in histological specimens and are associated with renal function

F. Genovese*; A. L. Reese-Petersen*,†; N. Sparding*,†; M. A. Karsdal*; V. Satrapova‡; D. Frausova‡; L. Bartonova§; E. Honsova§; V. Tesar‡; Z. Hruzkova‡

Nordic Bioscience, Herlev, Denmark; †Biomedical Sciences, Faculty of Health and Medical Science, University of Copenhagen, Copenhagen, Denmark; ‡Department of Nephrology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic; §Department of Pathology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic

Introduction: Renal fibrosis is a common hallmark of several indications of chronic kidney disease (CKD). Patients with more advanced and active fibrogenesis are more likely to progress to end-stage renal disease, a condition that presents a high risk of mortality and the dependance on dialysis or renal transplantation for survival.

The gold standard for evaluation of renal fibrosis is at the moment renal biopsy, a procedure presenting numerous drawbacks. It is therefore crucial to develop novel methods to detect and assess fibrosis in the renal tissue, in order to timely diagnose and stop the disease and to help the development of anti-fibrotic treatment.

Materials and Methods: Here, we quantified the concentration of novel biomarkers of fibrosis, namely a fragment of collagen type III generated by MMP-9 (C3M), reflecting interstitial matrix degradation, and a fragment of the α3 chain of collagen type VI C5 domain, representing collagen type VI formation, in serum and urine of patients with IgA nephropathy ($n = 49$) and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) ($n = 47$). Renal biopsies taken at the time of sample collections were evaluated for extent of fibrosis by an experienced pathologist.

Results: Both serological and urinary levels of PRO-C6 correlated with percentage of histological fibrosis (Spearman $r = 0.51$, $P < 0.0001$, and Spearman $r = 0.35$, $P = 0.0009$ respectively) and could separate patients with the most severe fibrosis stages as evaluated by the Banff score (ci0 and ci1 vs ci3, $P < 0.01$, and $P < 0.05$ respectively). Urinary levels of C3M inversely correlated with percentage of histological fibrosis (Spearman $r = -0.43$, $P < 0.0001$) and levels were the lowest in patients with the most advanced fibrosis stages (ci0 and ci1 vs ci3, $P < 0.05$).

Moreover, PRO-C6 in serum and urine gradually increased in increasing CKD stages, while urinary C3M gradually decreased in increasing CKD stages (Kruskal-Wallis $P < 0.0001$ for all markers).

Discussion: PRO-C6 and C3M were previously established as promising prognostic marker for adverse outcome in CKD in several independent cohorts. Here, we showed that these markers can actually reflect the burden of fibrosis and possibly the activity of the fibrogenic process happening in the kidneys.

PDE5 inhibitors and selective oestrogen receptor modulators exert anti-fibrotic synergy in *in vitro* and *in vivo* models of Peyronie's disease

M. M. Ilg*; M. Mateus*; W. Stebbeds†; U. Milenkovic‡; A. Muneer§; N. Christopher§; M. Albersen‡; D. J. Ralph§; S. Cellek*

Anglia Ruskin University, United Kingdom; †Cranfield University, United Kingdom; ‡Catholic University Leuven, Belgium; §University College London Hospital, United Kingdom

Introduction: Peyronie's disease (PD) is characterized by fibrosis in the penile tunica albuginea (TA) leading to pain, curvature and erectile dysfunction. Currently treatment is almost limited to surgery, highlighting the need for novel medication.

Materials and Methods: A novel high-throughput phenotypic screening assay measuring TGF-β1-induced myofibroblast transformation derived from human TA was developed. This assay was then used to screen FDA-approved drugs using In-Cell ELISA (ICE) method which revealed hits from two classes, selective oestrogen receptor modulators (SERMs) and PDE5 inhibitors (PDE5i). These were further confirmed using functional assays measuring contraction and ECM formation. The hits and their combination were investigated in vivo using an animal model of PD. Sprague-Dawley rats were divided in 5 groups: 1) TGF-β1 injection in the TA

(TGI) 2) vehicle injection, 3) TGI plus daily PDE5i (vardenafil), 4) TGI plus daily SERM (tamoxifen), 5) TGI plus daily combination of PDE5i and SERM. Five weeks after injection +/− treatments the rats were subjected to erectile function measurement with subsequent molecular analysis of the penis.

Results: The fibroblast identity of human TA-derived cells was confirmed. ICE was able to reproducibly quantify TGF-β1-induced transformation of fibroblasts to myofibroblasts. Five FDA approved drugs (three PDE5i and two SERMs) significantly inhibited TGF-β1-induced myofibroblast transformation in a concentration-dependent manner. The drugs were capable of decreasing collagen contraction, as well as ECM formation and synergised in inhibiting TGF-β1-induced myofibroblast transformation and collagen contraction. *In vivo* data revealed that TGF-β1 injection caused fibrosis in the penis of rats which resulted in erectile dysfunction, ECM accumulation and smooth muscle loss. These effects were prevented in the groups which received a PDE5i or SERM. The drug combination showed a synergistic effect on various hallmarks of fibrosis.

Discussion: An anti-fibrotic effect could be confirmed for PDE5i, SERM, and their combination *in vitro* and *in vivo* on a functional, histological and molecular level. The drugs synergise both *in vitro* and *in vivo*, suggesting a potential combination therapeutic approach for early phases of PD and possibly for other fibrotic diseases.

Proteoglycan-integrin interaction drive pathological cardiac remodelling

A. Romaine*,†; A. O. Melleby*,†; I. Sjaastad*,†; K. M. Herum*,†,‡; D. Gullberg§; G. Christensen*,†

*Institute for Experimental Medical Research, Oslo University Hospital and University of Oslo, Norway; †Center for Heart Failure Research & K.G. Jebsen Centre for Cardiac Research, Oslo University Hospital, Oslo, Norway; ‡Biotech Research & Innovation Centre, University of Copenhagen, Copenhagen, Denmark; §Department of Biomedicine, University of Bergen, Bergen, Norway

Introduction: Heart failure is a condition with very poor prognosis affecting 26 million people globally, with no current effective therapy. A key driver of heart failure is pressure overload, which results in cardiac fibrosis and a deleterious stiffening of the left ventricle. Proteoglycans and integrins represent novel members of the mechanosensing apparatus of the heart, crucial to detecting alterations in the mechanical stress the heart is exposed to. Determining the key mechanoreceptors involved in pathological cardiac remodelling is critical to identifying new therapeutic targets.

Materials and Methods : We generated genetically modified mice lacking expression of syndecan-4 (SDC4), integrin α11β1 (Itgα11) and a double knockout strain. Mice were subjected to aortic banding to induce cardiac pressure overload and phenotyped over a period of 2 weeks. In vitro experiments were conducted on a mouse fibroblast cell line (NIH 3T3) to elucidate the functional effects of SDC4 and Itgα11 expression on fibrosis.

Results and Discussion : SDC4$^{-/-}$ and Itgα11$^{-/-}$ mice still developed cardiac hypertrophy and fibrosis following surgical induced pressure overload comparable to wild-type (WT) mice at the 2 week time-point; however, SDC4$^{-/-}$Itgα11$^{-/-}$ double knockout mice showed reduced pathological remodelling, including reduced fibrosis. We attribute this protective phenotype to a functional overlap between SDC4 and Itgα11 receptors. Through in vitro experiments, we determined a reciprocal pattern of expression between these receptors under different stimulations, gene silencing and overexpression assays, suggesting single targeting of these receptors is ineffective. Further, we suggest a novel mechanism by which SDC4 regulates the surface expression of Itgα11 mediated through endosomal trafficking. Together our results indicate that the dual targeting of these receptors may be of therapeutic benefit to patients at risk of developing pressure overload induced heart failure.

Mutations in the COL1A1 and COL1A2 genes associated with osteogenesis imperfecta (OI) types I or III

A. Augusciak-Duma*; J. Witecka*; A. L. Sieron*; A. Galicka†

*Department of Molecular Biology and Genetics; School of Medicine in Katowice; Medical University of Silesia, Katowice, Poland; †Department of Medical Chemistry, Medical University of Bialystok, Poland

Introduction: *Osteogenesis imperfecta* (*OI*) is a heterogenic, connective tissue and extracellular matrix related disorder, manifested by bone fragility, with autosomal dominant, rarely recessive, inheritance and $1/10^4$ to $1/3 \times 10^4$ prevalence. Although over 85% of *osteogenesis imperfecta* (*OI*) cases are associated with mutations in the procollagen type I genes (*COL1A1* or *COL1A2*), no hot spots for the mutations were associated with particular clinical phenotypes.

Materials and Methods: Eight patients that were studied here, diagnosed with *OI* by clinical standards, are from the Polish population with no ethnic background indicated. DNA for analysis was isolated from blood samples and was PCR amplified followed by DNA sequencing on ABI Prism $3130 \times$ l sequencer.

Results : Previously unpublished mutations were found in six out of those eight patients. Genotypes for polymorphisms (Sp1—rs1800012; and PvuII—rs412777), linked to bone formation and metabolism were determined. Mutations

were found in exons 2, 22, 50 and in introns 13 and 51 of the *COL1A1* gene. In *COL1A2*, one mutation was identified in exon 22.

Discussion: Mutation locations analysed here did not point directly to type of *OI*. The resulting changes in the encoded amino acids are not sufficient for predicting how severe the disorder will be. The consistence of the glycine codon changes to the cysteine codon (at position 403 in patient 19/F) correlates with commonly accepted Gly to Xxx substitutions resulting in a more severe type III or a lethal type II of *OI*. In addition, the possible exon skipping effect of I51 mutation at the 4248 + 1 position could explain the more severe type III phenotype manifested by a tremendous number of bone fractures. Results obtained by detection of mutations in genes encoding procollagen type I in patients diagnosed with either type I or III *OI* revealed that the causative mutations might occur anywhere in both genes.

Substrate Specificity of Collagen Prolyl 4-Hydroxylases

A. M. Salo*; P. Rappu†; K. Drushinin*; J. Käpylä†; J. Heino†; K. I. Kivirikko*; J. Myllyharju*

Biocenter Oulu and Faculty of Biochemistry and Molecular Medicine, FI-90014 University of Oulu, Finland; †Department of Biochemistry, FI-20014 University of Turku, Finland

Introduction: Collagen prolyl 4-hydroxylases (C-P4H) catalyze the formation of 4-hydroxyproline in collagenous -XPG- sequences. Enzyme tetramers consist of two identical catalytic alpha subunits and protein disulphide isomerases as beta subunits. P4HA1, P4HA2 and P4HA3 encode the alpha subunit genes. Mutations in P4HA1 have been described in congenital disorder manifesting as early-onset joint hypermobility, joint contractures, muscle weakness, bone dysplasia and high myopia. Mutations in P4HA2 cause myopia. In this work, we show collagen sequence specificity of P4H isoforms.

Materials and Methods: We used prolyl 4-hydroxylase mutant mouse (P4 ha2$^{+/-}$; P4 ha2$^{+/-}$, P4 ha2$^{-/-}$; P4 ha1$^{+/-}$; P4 ha2$^{-/-}$) skin and P4 ha1$^{-/-}$ embryonic fibroblasts to extract collagen for tandem mass spectrometry in order to identify proline residues that have affected hydroxylation. Results were confirmed by activity assay using (XPG)5 peptides with different X-position amino acids and purified recombinant C-P4H enzymes.

Results: Extracted collagen was analysed by mass spectrometry. Results were confirmed by in vitro activity assays. Data indicated that deletion of P4 ha1 leads to substantial decrease in 4-hydroxylation of prolines. Deletion affects many different hydroxylation sites. Data showed also that C-P4H-I is very poor in catalyzing hydroxylation of prolines that follow aspartate or glutamate, whereas C-P4H-II can efficiently hydroxylate those. In addition, absence of C-P4H-II leads to slight underhydroxylation when X-position amino acid is uncharged serine, glutamine or threonine. In contrast, deletion leads to slight overhydroxylation when X-position amino acid is aliphatic. In conclusion, we noticed a role of X-position amino acid to adjacent proline to be hydroxylated.

Discussion: Our results indicate that multiple isoforms of C-P4H are needed as single isoform cannot fully hydroxylate collagen. We observed clear sequence specific prolyl 4-hydroxylation by different isoenzymes. Data explain some of the phenotypes observed in mouse models and human prolyl 4-hydroxylase-related diseases. Data suggest that catalytic domain of C-P4H has selectivity in hydroxylation sites.

Bone-specific abnormal collagen post-translational chemistry and cross-linking causing bone fragility in Bruck Syndrome caused by compound heterozygous PLOD2 mutations

C. Gistelinck*; M. Weis*; J. Rai*; P. H. Byers†; D. R. Eyre*

Department of Orthopaedics and Sports Medicine, University of Washington, Seattle, WA, United States of America; †Department of Pathology and Medicine, University of Washington, Seattle, WA, United States of America

Introduction: Bruck syndrome (BS) is a congenital disorder characterized by joint flexion contractures, skeletal dysplasia and increased bone fragility, showing strong clinical overlap with Osteogenesis Imperfecta (OI). On a genetic level, BS is caused by bi-allelic mutations in either the *FKBP10* or the *PLOD2* gene. *PLOD2* encodes the lysyl hydroxylase 2 (LH2) enzyme which is responsible for the hydroxylation of lysyl residues in fibrillar collagen telopeptides. This modification is essential for allowing collagens to form stable intermolecular cross-links in the extracellular-matrix. Bone collagen has a unique pattern of cross-linking that is required for bone strength, resistance to microdamage and crack propagation and also a normal ordered pattern of mineral nanocrystals in the collagen fibrils. To date, no direct studies of human bone from BS caused by *PLOD2* mutations have been reported. We present here results from a case of BS in a 4-year-old Caucasian patient, caused by compound heterozygous mutations in *PLOD2*.

Materials and Methods: Bone tissue was collected during surgery, and bone collagen was biochemically characterized using SDS-PAGE, cross-link analysis and peptide mass-spectrometry.

Results: The patient's bone showed diminished hydroxylation of type I collagen telopeptide lysines, while hydroxylation at helical sites was unaltered. Consequently, mature trivalent cross-links, which depend on the presence of telopeptide hydroxylysines, were shown to be greatly reduced. Mass-spectrometry identified abundant allysine aldol dimeric cross-links in the patient's bone, which are not normally present in bone but are a feature skin collagen. SDS-PAGE further illustrated a skin-like migration pattern of the patient's extracted bone collagen, with more prevalent β-dimers and γ-trimers. Type II collagen cross-linked peptides from the patient's urine were also analyzed. In contrast to bone type I collagen, the results showed a normal telopeptide lysine hydroxylation of cartilage type II collagen.

Discussion: Taken together, these findings shed light on the complex mechanisms that control the unique posttranslational chemistry and cross-linking of bone collagen, and that when defective can cause a brittle bone disorder.

Characterization of Bone Extracellular Matrix Produced By Recql4-Deficient Osteoblasts

T. Gritsaenko*; V. Pierrefite-Carle*; J.-M. Guigonis*; T. Lorivel†; F. Orange‡; C. Cros*; G. F. Carle*; S. Santucci-Darmanin*

*UMR E-4320 TIRO-MATOs CEA/DRF/BIAM, Université Nice Sophia-Antipolis, France; †Institut de Pharmacologie Moléculaire et Cellulaire, UMR 7275, CNRS, Université Nice Sophia-Antipolis, Valbonne; ‡Centre Commun de Microscopie Appliquée (CCMA), Université Nice Sophia-Antipolis, France

Introduction: Bone is a complex and mineralized tissue under permanent remodeling, in which the main cellular actors are osteoblasts (OB), synthesizing the mineral matrix, osteoclasts (OCL), in charge of bone resorption, and osteocytes (OST), acting as mechanosensors. The bone extracellular matrix (ECM) is a dynamic network of molecules secreted by OB and OST which in turn regulate the behavior of all bone cells by modulating their proliferation, differentiation and function. As an example, senescent OB create a defective microenvironment through an altered secretome, which in turn stimulates OCL function. This observation highlights the connection between OB secretome, ECM and bone cells behavior. Our laboratory is studying the *recql4*$^{-/-}$ murine model potentially associated with altered OB secretome. RECQL4 is a DNA helicase involved in genomic stability and its dysfunction has been associated with cellular senescence. Aiming to decipher the mechanisms underlying bone loss in our model, we started investigating the interactions between the OB-produced mineralized ECM and bone cells.

Materials and Methods: The femur microarchitecture from *recql4*$^{-/-}$ and *recql4*$^{+/-}$ control mice was analyzed by micro-computed tomography. Primary OB were isolated from those mice and used to synthesize bone ECM *in vitro* which were examined for protein and mineral composition as well as for ultrastructure using different approaches such as calcium staining, proteomic analysis and scanning electron microscopy.

Results: *recql4*$^{-/-}$ mice exhibit a premature bone aging phenotype. Bone matrices produced by *recql4*$^{-/-}$ OB tend to be less mineralized than those from control OB. A first set of proteomic analyses revealed 3 proteins missing from the mutant ECM and known to be involved in osteogenesis regulation: one that seems to be required for matrix mineralization by OB; another that is involved in osteoblastogenesis regulation and a coupling factor linking bone resorption and bone formation.

Discussion: Depletion of any of the 3 candidate proteins we have identified might lead to a phenotype observed in our mouse model. Experiments to confirm these findings are in progress. The subsequent step will be to compare differentiation and function of osteoclasts on mineralized matrices synthesized by *recql4*$^{-/-}$ and *recql4*$^{+/-}$ OB.

Towards an atlas of matrisome regulation in cancer

V. Izzi*; J. Lakkala*; R. Devarajan*; R. Heljasvaara*,†; T. Pihlajaniemi*

*Center for Cell-Matrix Research and Biocenter Oulu, Faculty of Biochemistry and Molecular Medicine, University of Oulu, Finland; †Centre for Cancer Biomarkers (CCBIO), Department of Biomedicine, University of Bergen, Bergen, Norway

Introduction: Alterations in the quantitative and qualitative content of the tumor microenvironment are of primary importance in the neoplastic process, so much so as to have been recognized since the pioneering theory of the "bad seed in bad soil" formulated by Paget in 1889. Nowadays, the complex network of extracellular matrix (ECM) components, growth factors, cytokines and enzymes is better recognized as a whole "omic" world (defined as the "matrisome"), regulated by an intricated and yet poorly understood network of transcriptional programs. Based on the enormous importance of matrisome regulation in cancer, we are developing a "first-of-its-kind" integrated approach to: 1) catalogue mutational and variational events in the cancer matrisome, 2) define which matrisome genes are specific to any given tumor and, 3) unravel transcriptional regulatory pathways operating on matrisome genes.

Materials and Methods: Complete mutational, variational, transcriptional and clinical data from The Cancer Genome Atlas (TCGA) PANCAN cohort were downloaded, filtered for quality and completeness and used for further analyses, for a total of 822 matrisome genes assessed in 10487 patient

samples representing 32 human tumors. Various profiling and clustering algorithms, together with a custom-made gene regulatory network (GRN)-inferring software, were used for the analyses.

Results: Different tumors vary notably in the amount of mutations and variations per each given class of matrisome genes, but also show tissue- and system-of-origin similarities for what concerns the expression of these genes, so much so that clusters of neoplasms (with important differences in their clinicopathological features) can be inferred. Furthermore, common GRNs seem to characterize different neoplasms belonging to the same cluster, evidencing a relatively small amount of master transcriptional programs which similarly regulate matrisome genes in different cancers.

Discussion: Our results represent, to date, the most complete effort at characterizing and understanding the molecular biology of cancer matrisome, and offer new insights into its regulatory mechanisms with significant consequences for clinical and pharmacological research.

Isolation, culture and characterization of cells from human abdominal aortic aneurysm

M. Lesiak*; K. Bugaj*; A. Fus*; D. Ziaja†; M. Sznapka†; K. Ziaja†; W. Kuczmik†; A. L. Sieroń*

*Department of Molecular Biology and Genetics, School of Medicine in Katowice, Medical University of Silesia in Katowice, Poland; †Department of General Vascular Surgery, Faculty of Medicine in Katowice, Medical University of Silesia in Katowice, Poland

Introduction: Abdominal aortic aneurysm (AAA) is common and asymptomatic life-threatening illness. It comes into existence as a result of abdominal aortic walls dissection, which leads to blood vessel rupture and patient's death in great measure. AAA is a multifactorial clinically heterogenic disease; therefore, an indication of one main factor causing the aneurysm is difficult.

Materials and Methods: The aim of the study was to standardize the cell culture methods for cells obtained from AAA specimens and determination of their phenotypes. The cells were isolated from separated inner, external and median layer of each AAA fragment. The cells were compared to control commercial cell types from health human abdominal aortas. Isolated cells were cultured in an appropriate standard culture media. Subsequently, the cell cultures were analysed by flow cytometry, immunochemistry and macroscopic methods.

Results: Cells from the inner layers of aorta of all patients needed the shortest time to reach primary confluence. The cells from external layers of all patients presented the longest time to reach primary confluence. The shortest time to reach confluence after the recovery from the banking in liquid nitrogen presented the cells from the middle layers of all patients. In all the layers only small fractions of cells presented layer specific cell type markers. Majority of the cells from every layer were the cells positive for CD90 considered as fibroblast marker. The cells from the layers did not show the expected morphology, such as for endothelium in the inner layer, smooth muscle in the middle and fibroblasts in the external layer.

Discussion: Abdominal aortic aneurysm is a very important life-threatening health problem. Therefore, finding a specific prognostic marker, which is associated with the presence and progression of AAA, is urgently crucial. The studies here have confirmed that in the wall of the AAA, there is a lack of specific cell type composition in comparison with the normal abdominal aorta wall. Further studies are planned on the cells molecular pathobiology in AAA as well as the cross-talk between the ECM and the cells in AAA.

Andrographolide inhibits osteoclastogenesis by suppressing ERRα and prevents ovariectomy and obesity induced bone loss

M. Guan; T. Huang; M. Yang

Center for Human Tissues and Organs Degeneration, Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences

Introduction: Osteoclasts play pivotal roles in the regulation of bone homeostasis. Atypical increases in osteoclast activity cause an imbalance in bone remodeling that leads to bone diseases. Estrogen-related receptor α (ERRα) is an orphan nuclear hormone receptor that regulates various metabolic processes. Osteoclasts undergo metabolic adaptations to meet the increased bio-energetic demands of the energy consuming process of bone resorption. Glutaminase converts glutamine to glutamate that is further catabolized through TCA cycle for ATP production. We hypothesized that ERRα coordinates with PGC-1β to mediate such metabolic transition that advances osteoclast differentiation. Andrographolide, a diterpene lactone compound extracted from the leaves of Acanthaceae paniculata, might suppress osteoclast differentiation by modulating ERRα activity.

Materials and Methods: Bone marrow monocytes were isolated and differentiated into osteoclasts. Cells were infected with ERRα or glutaminase-expressing lentivirus, and simultaneously treated with or without andrographolide. Luciferase reporter assay, real-time PCR and Western blot were used to analyze the transcriptional regulation and expression levels. Ovariectomized female mice and high-fat diet-treated male mice were orally gavaged

with andrographolide daily for 4 weeks. Bone analyses were performed by micro-computed tomography and histochemistry.

Results: ERRα coordinated with PGC1β to mediate glutaminase expression and then facilitated osteoclast differentiation. Andrographolide suppressed osteoclast differentiation by acting as an ERRα modulator. Furthermore, andrographolide suppressed *in vivo* osteoclast formation and prevented estrogen deficiency and high-fat diet-induced bone loss.

Discussion: These data provide evidence for an underlying mechanism by which ERRα/PGC1β enhances osteoclastogenesis and affects bone homeostasis. This study also highlights that pharmacological inhibition of ERRα may be further developed into novel treatments for human bone disorders such as osteoporosis.

Regulatory role of collagen XIII in breast cancer

C. Henriksson; N. Petrelius; R. Devarajan; A. Heikkinen; J. Koivunen; S. Kauppila; R. Winqvist; F. Wärnberg; M. Sund; R. Heljasvaara; T. Pihlajaniemi

Faculty of Biochemistry and Molecular Medicine, University of Oulu, Finland

Introduction: Breast cancer is significantly influenced by the local microenvironment consisting of different types of cells and the extracellular matrix (ECM). Collagens form a major ECM protein family and are often produced in excess by tumour cells. Collagen XIII (ColXIII) belongs to the subfamily of membrane-associated collagens with interrupted triple helices (MACITs). ColXIII is highly expressed in epithelial and mesenchymal tumours, and it regulates cell adhesion and signaling. The Cancer Genome Atlas (TCGA) analysis suggested that ColXIII is upregulated in solid tumours. In the current study, we have explored the expression and specific roles of ColXIII particularly in breast cancer.

Materials and Methods: Bioinformatic and Kaplan-Meier log rank analysis was performed for overall survival and relapse-free survival. Human breast cancer samples were stained for ColXIII and scored for the intensity and localization. ColXIII knockout mice were used to study the tumour development in vivo. Human breast cancer cell lines were used to determine the role of ColXIII in vitro, and siRNAs were used to knock down ColXIII expression.

Results: Bioinformatic analysis showed that high ColXIII expression associates significantly with the poor survival particularly in estrogen receptor (ER)-negative patients. Immunohistochemical stainings of human breast cancer tissues showed that ColXIII is abundantly expressed in tumour cells. In the MMTV-PyMT mouse mammary carcinoma model, lack of Col13a1 gene led to significantly reduced tumour cell proliferation and tumour growth in a later stage of carcinogenesis in comparison with the control PyMT mice. Moreover, ColXIII expression was highly upregulated in the triple negative human breast cancer cell lines and siRNA-mediated knockdown of ColXIII significantly reduces the proliferation and viability of these cells.

Discussion: Our data show that ColXIII is actively involved in the breast tumourigenesis. Further studies will be conducted to identify the underlying molecular mechanisms through which ColXIII regulates the breast cancer progression.

Investigating drivers of the tumour matrisome, and their role in disease progression

O. M. T. Pearce*; S. Nichols*; R. Delaine-Smith*,†; R. R. Jones*; E. Maniati*; R. Rootizalab*; J. Mackerodt*; M. M. Knight†; F. R. Balkwill*

**Centre for Cancer Inflammation, Barts Cancer Institute, QMUL, Charterhouse Square, London, EC1M 6BQ; †School of Engineering and Materials Science, QMUL, Mile End Road, London, E1 4NS*

Introduction: In our recently published work, we identified a tumour matrisome composition that we call the "Matrix Index," which defines a matrisome that associates with poor prognosis, and is common to many cancer types. Here, we present our work to understand how the matrix index is generated, and the interplay between tumour progression, invasion, and tissue remodelling.

Materials and Methods: We have constructed 3D tumour models made from primary human adipocytes, fibroblasts, and mesothelial cells. Tumour cell lines can be introduced either to the 3D gel surface to study metastasis, or implanted into the gel to study late-stage disease. These models are useful for investigating matrix remodelling, tumour progression, and invasion.

Results: We find platelets stimulate a matrisome response in malignant cells, which aids proliferation and invasion of the tumour. Using small-molecule inhibitors, we find these matrix index molecules are controlled through the TGFB and hedgehog signalling pathways.

Discussion: Platelet-activated malignant cells contribute to the establishment of the matrix index, fibroblast activation, tumour progression, and invasion within our 3D tissue models. Tumour expressed matrix can be suppressed using TGFB receptor and hedgehog signalling inhibitors, which in turn blocks tumour progression. Therefore, blocking tumour matrix production may inhibit tumour progression in combination with clinically used cancer therapeutics.

CANT1 role in proteoglycan synthesis: an *in vivo* study with a mouse model of Desbuquois dysplasia type 1

C. Paganini*; R. Costantini*; L. Monti*; C. Gramegna Tota*; A. Lundari*; M. Biggiogera†; J. M. Schwartz‡; C. Huber§; V. Cormier-Daire§; B. Gibson¶; K. A. Pirog¶; M. Briggs¶; A. Forlino*; A. Rossi*

*Department of Molecular Medicine, Unit of Biochemistry, University of Pavia, Italy; †Department of Biology & Biotechnology, University of Pavia, Italy; ‡Faculty of Biology, Medicine and Health, University of Manchester, United Kingdom; §Department of Genetics and INSERM UMR1163, Hôpital Necker Enfants Malades, Paris, France; ¶Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom

Introduction: Mutations in *CANT1* gene cause Desbuquois dysplasia type 1 (DBQD1) a recessive chondrodysplasia characterized by growth retardation, multiple dislocations and hand deformities. *CANT1* encodes for a calcium activated nucleotidase of the ER/Golgi that preferentially hydrolyses UDP; due to its function and localization a role in proteoglycan (PG) metabolism has been inferred. To investigate this hypothesis and CANT1 role in DBQD we have generated a *Cant1* knockout mouse (*Cant1*$^{-/-}$ mouse).

Materials and Methods: Morphological analysis of mice was performed by skeletal double staining with alcian blue and alizarin red and by X-ray. Rib chondrocytes were metabolically labelled with ^{35}S-sulfate to analyse PG synthesis and glycosaminoglycan hydrodynamic size. Glycosaminoglycan sulfation was determined by HPLC analysis. PG secretion was studied by pulse-chase ^{35}S-sulfate labelling, and morphology of chondrocytes was investigated by TEM. Microarray of ER stress markers was performed on RNA extracted from rib cartilage. Expression level of BiP was confirmed by Western blot analysis, while the presence of spliced form of Xbp1 (Xbp1s) was studied by RT-PCR.

Results: *Cant1*$^{-/-}$ mice were smaller and showed reduced skeletal growth compared with wild-type animals reproducing the growth defects of patients. In *Cant1*$^{-/-}$ chondrocytes reduced PG synthesis was demonstrated and glycosaminoglycans showed reduced hydrodynamic size and oversulfation compared with wild-types. Pulse-chase experiment demonstrated reduced PG secretion in mutant cells compared with controls and in *Cant1*$^{-/-}$ chondrocytes dilated vacuoles containing electron dense material were observed by TEM. RNA microarray demonstrated no overexpressed ER stress markers in *Cant1*$^{-/-}$ cartilage. Protein level of BiP was normal in *Cant1*$^{-/-}$ cells and Xbp1s was not present in *Cant1*$^{-/-}$ cartilage.

Discussion: *Cant1*$^{-/-}$ mouse is a useful animal model to study DBQD1 being reminiscent of the patients' phenotype. CANT1 affects proteoglycan synthesis at different levels including glycosaminoglycan chain length, sulfation and secretion in the extracellular matrix. The presence of huge vacuoles in *Cant1*$^{-/-}$ chondrocytes suggests CANT1 involvement in protein retention. However, intercellular retention in mutant cells does not cause ER stress since ER stress markers such as BiP and Xbp1s are normal in *Cant1*$^{-/-}$ chondrocytes and cartilage.

ADAMTS7 substrate and cleavage site specificity

R. de Groot*; C. Monseur†; A. Colige†; S. Santamaria*; J. T. B. Crawley*

*Imperial College London, Department of Medicine, Hammersmith Hospital Campus, London, United Kingdom; †University of Liège, Laboratory of Connective Tissues Biology, Liège, Belgium

Introduction: The metalloprotease ADAMTS7 has been implicated in the aetiology of coronary artery disease. However, little is known about its physiological function or its substrate specificity. We aimed to characterise the biochemical properties of ADAMTS7 through identification of proteolytic substrates and their respective cleavage site(s).

Materials and Methods: ADAMTS7 variants and a short LTBP4 fragment were expressed in HEK293(T) cells and purified using anion exchange/gel filtration and anti-flag Ab affinity purification respectively. N-terminal sequencing was outsourced. iTRAQ-TAILS was performed using conditioned medium of fibroblasts co-cultured with HEK cells that expressed either ADAMTS7 or mutated (E389Q) inactive ADAMTS7.

Results: Analysis of recombinant ADAMTS7 by Western blot (WB) identified both latent (+ prodomain) and active (- prodomain) forms, as well as a lower molecular weight (MW) band that was a product of autolysis. Mutagenesis of the active site or prevention of ADAMTS7 activation by furin abolished autolysis. N-terminal sequencing of the autolytic product revealed proteolysis of the Glu732-Ala733 bond in the spacer domain. Mass spectrometry confirmed this and identified a second autolytic cleavage nearby (Glu729-Val730). Mutagenesis of Glu729 and Glu732 to Ala almost completely abolished autolysis, confirming these as major autolytic cleavage sites. These results suggest ADAMTS7 scissile bond specificity favours P1 Glu and small hydrophobic residue at P1'. This information was utilised in the interpretation of iTRAQ TAILS data, leading to the identification of cleavages of Glu-Ala and Glu-Val in latent transforming growth factor β binding proteins, LTBP4 and LTBP3, respectively, in the linker region between the first EGF-like domain and the hybrid domain. These proteins play roles in fibrillin microfibril biology. WB of a recombinant LTBP4 fragment incubated with ADAMTS7 or inactive ADAMTS7 (E389Q) confirmed that LTBP4 is susceptible to ADAMTS7 proteolysis. Proteolysis could be inhibited

by TIMP3, a known ADAMTS inhibitor, as well as by small-molecule metalloprotease inhibitors, marimastat and batimastat.

Discussion: We identified Glu-Ala/Val as a scissile bond of ADAMTS7 and LTBP4 as a potential substrate. Further studies are underway to ascertain whether LTBP3/4 are physiological substrates and if so, what the consequences of ADAMTS7 proteolysis are.

Mutations in the collagen I prolyl 3-hydroxylation complex cause altered cellular homeostasis

B. Roberta*; G. Nadia*; A. Gagliardi†; C. Carnemolla†; S. Simone‡; B. Marco§; L. Bini†; L. Bianchi†; R. Antonio*; F. Antonella*

*Department of Molecular Medicine, Biochemistry Unit, University of Pavia, Pavia, Italy; †Department of Life Sciences, University of Siena, Siena, Italy; ‡Istituto di Genetica Molecolare, CNR, Pavia, Italy; §Department of Biology and Biotechnology, University of Pavia, Pavia, Italy

Introduction: The brittle bone disease osteogenesis imperfecta (OI) is a collagen-related disorder associated with dominant, recessive or X-linked transmission. Among the recessive forms, OI types VII, VIII, and IX are due to mutations in *CRTAP*, *P3H1*, and *PPIB* genes, respectively, coding for the components of the endoplasmic reticulum (ER) complex that catalyses 3-hydroxylation of type I collagen α1(I)Pro986. In dominant OI, due to mutations in type I collagen genes, we recently demonstrated the presence of an altered cytoskeleton and of an ER retention of overmodified collagen, causing cellular stress that contributes to OI pathogenesis. Here, we investigated the effect of mutations in the *CRTAP*, *P3H1*, and *PPIB* genes on cellular homeostasis in primary fibroblasts from OI patients.

Materials and Methods: Cytoskeleton and nucleoskeleton asset were investigated by functional proteomic and confocal analyses. ^{3}H proline labelled type I collagen was analysed by electrophoresis. ER morphology was evaluated by transmission electron microscopy. The activation of unfolded protein response (UPR), autophagy and apoptosis were determined by Western blot and FACS. Cells were treated with 4-phenylbutyrate (4-PBA) and the effect was evaluated by Western blotting. General protein secretion was determined by labelling with ^{35}S-L-methionine and ^{35}S-L-cysteine.

Results: Altered expression/distribution of lamin A/C and cofilin-1 revealed an aberrant organization of nucleus and cytoskeleton in mutant fibroblasts. The overmodified collagen, synthesized by OI patients cells and partially intracellular retained, caused ER enlargement and the activation of the PERK branch of the UPR leading to an increased cell death. The treatment with 4-PBA reduced UPR, ER cisternae size and apoptosis thanks to its stimulatory effect on general protein secretion and autophagy.

Discussion: We have shown that recessive OI forms with mutations in the collagen I prolyl 3-hydroxylation complex share common features with classical dominant OI, such as cytoskeleton alteration and cellular response to altered collagen molecules. Furthermore, in both cases, 4-PBA rescued the altered homeostasis caused by the presence of overmodified collagen I, demonstrating that cellular ability to cope with stress can be a valid target for OI treatment.

Generation and characterization of an animal model of chondrodysplasia with joint dislocations gPAPP type

R. Costantini*; L. Monti*; C. Paganini*; A. Lundari*; S. Sonntag†; D. Shmerling†; A. Forlino*; A. Rossi*

*Department of Molecular Medicine, Unit of Biochemistry, University of Pavia, Italy; †PolyGene AG, Rümlang, Switzerland

Introduction: Chondrodysplasia with joint dislocations gPAPP type is a recessive skeletal disorder caused by mutations in the *IMPAD1* gene encoding for gPAPP, a Golgi-resident 5'-phosphoadenosine 3'-phosphate (PAP) 3'-phosphatase that hydrolyzes PAP, the by-product of sulfotransferase reactions, to AMP and phosphate. This enzyme is involved in glycosaminoglycan (GAG) sulfation and its function has been confirmed in Impad1 knockout mice (PNAS, 105, 11605-12). Mutant mice are lethal at birth precluding the study of IMPAD1 in post-natal skeletal development which is important in chondrodysplasia gPAPP type because patients do not have a lethal phenotype. For this reason we have generated a conditional knock-in mouse that reproduces a patient's missense mutation.

Materials and Methods: Morphological observations were performed by X-rays and skeletal staining with alcian blue and alizarin red. RNA was extracted from skin of newborn mice and *Impad1* expression was evaluated by qPCR. Cartilage proteoglycan sulfation analysis was performed by HPLC disaccharide analysis.

Results: The first Impad1 conditional knock-in for a missense mutation (Asp175Asn) reported in a patient was generated. Unexpectedly, the phenotype of mutant homozygous mice was lethal at birth with severe hypoplasia of the skeleton. Biochemical analysis of cartilage from wild-type, knock-in and knockout mice demonstrated that the amount of non-sulfated disaccharide relative to the total amount of disaccharides was significantly increased in knock-in mouse, equally to the knockout mouse. Since the knock-in mouse shows the same phenotype as the knockout, we analyzed the molecular basis of the lethal phenotype. Potential alternative

splicing of the targeted allele were investigated by RT-PCR: in the wild-type a transcript corresponding to the whole coding sequence was detected, while in the homozygous mutants two different Impad1 transcripts lacking exon 2 or exons 2-3 were observed.

Discussion: Results demonstrated that the mouse lethality was due to alternative splicing of the targeted allele caused by the strategy used in the set-up of the conditional knock-in targeting vector. For this reason, a new knock-in mouse strain has been generated using a different gene targeting vector and is currently under investigation.

Using patient-derived induced pluripotent stem cells to model multiple epiphyseal dysplasia

S. Woods*; P. Harley*; J. Soul*; N. Kamprom*; N. Bates*; Q. Wang*; G. Mortier†; T. Hardingham*; S. J. Kimber*

*University of Manchester, Manchester, United Kingdom; †Antwerp University Hospital and University of Antwerp, Antwerp, Belgium

Introduction: Multiple epiphyseal dysplasia (MED) is a chondrodysplasia characterised by delayed epiphyseal endochondral ossification, resulting in disproportionate short stature and early-onset osteoarthritis. MED can be caused by heterozygous mutations in COMP, MATN3, COL9A1, COL9A2 and COL9A3, or bi-allelic mutations in SLC26A2. Human induced pluripotent stem cells (hiPSCs) are reprogrammed somatic cells which can differentiate to form all body tissues and have excellent potential for tissue regeneration as well as providing models of human disease. Our aim is to generate an *in vitro* hiPSC model of growth-plate development in order to better understand MED.

Materials and Methods: HiPSCs were generated from peripheral blood mononuclear cells (PBMCs) of 3 related MED individuals who are heterozygous for a MATN3 p.Val194Asp mutation (V194D) and 4 healthy controls. HiPSCs were differentiated to growth-plate-like chondrocytes via an iPSC-MSC-like intermediate, followed by TGFβ3$^+$ BMP2-induced chondrogenic pellet culture for 21 days.

Results: Healthy and V194D hiPSCs were able to differentiate to iPSC-MSCs which displayed typical MSC morphology, expressed MSC markers (CD90, CD105, CD44 and CD73) and were capable of generating cartilage and bone. After 21 days in TGFβ3$^+$ BMP2-containing medium, V194D chondrogenic pellets were significantly larger in size, stained more strongly for cartilage-associated sulphated glycosaminoglycans (alcian blue and Safranin O), and expressed significantly higher levels of SOX9, COL2A1 and ACAN transcript. RNA-seq validated these differences and pathway analysis of V194D vs healthy identified enrichment of terms such as "dwarfism" and "skeletal system development." Immunohistochemistry and confocal co-localisation analysis showed matrilin-3 was retained within the ER of the V194D mutant pellets; interestingly, we observed little evidence of ER stress.

Discussion: These data suggest V194D mutant cells respond differently during TGFβ3$^+$ BMP2-induced chondrogenesis, which may be caused by altered matrix regulation of growth factor availability. This model provides novel insight into MED disease pathogenesis and will enable screening of pharmaceutical products.

Connecting muscle and matrix: clinical and molecular characterization of six new families with myopathic Ehlers-Danlos syndrome

T. Dhooge; S. Delbaere; D. Syx; S. Symoens; F. Malfait

Center for Medical Genetics Ghent, Ghent University Hospital, Ghent University, Belgium

Introduction: Collagen XII, encoded by the *COL12A1* gene, is a fibril associated collagen with interrupted triple helices (FACIT) and modulates collagen organization by interacting with fibrillar collagens and extracellular matrix (ECM) proteins. Recently, *COL12A1* mutations were identified in patients displaying a novel overlap syndrome involving muscle and connective tissue, coined "myopathic Ehlers-Danlos syndrome (mEDS)." While heterozygous *COL12A1* mutations result in a relatively mild phenotype resembling collagen VI-associated Bethlem myopathy, a homozygous loss-of-function mutation in two siblings results in a more severe congenital mEDS phenotype. The small number of diagnosed patients limits thorough investigation of this newly identified syndrome.

Materials and Methods: We selected a cohort of molecularly unresolved EDS patients with signs of myopathy and sequenced *COL6A1, COL6A2, COL6A3* and *COL12A1*, using next-generation gene panel sequencing.

Results: We identified seven individuals from five families with a heterozygous *COL12A1* mutation and one patient with compound heterozygous *COL6A1* mutations, involving a nonsense and a splice-site mutation resulting in a frameshift. The *COL12A1* mutations comprised one arginine-to-cysteine and four splice-site mutations skipping exon 52, 53, 54 and 56 respectively. Important clinical symptoms included joint hypermobility ($n = 7/8$), hypotonia ($n = 5/8$) and contractures ($n = 5/8$). The phenotype of the *COL6A1*-mutant patient clinically resembled that of the *COL12A1*-mutant patients. In the ECM of skin fibroblasts of the *COL6A1*-mutant patient, immunocytochemistry

revealed a near-complete absence of collagen VI and V. Western blotting showed slightly increased tenascin-X secretion. In *COL12A1*-mutant patient fibroblasts, normal levels of secreted collagens XII, VI and V were observed. Some intracellular accumulation of collagen XII was seen in patients with exon skip 52 and 53, respectively, along with reduced decorin secretion. Secreted tenascin-X was diminished in all *COL12A1*-mutant fibroblasts.

Discussion: In conclusion, we expand the clinical and mutational spectrum of the recently delineated "myopathic EDS" with seven individuals from five families. We report a novel *COL12A1* arginine-to-cysteine substitution, and four new exon-skipping mutations clustering to the same region, with mutation-dependent abnormalities in the skin ECM. Additionally, we report a patient with a clinically similar mEDS phenotype with compound heterozygous loss-of-function *COL6A1* mutations. Further studies are necessary to understand the pathogenic mechanisms underlying these overlapping phenotypes combining muscle and connective tissue defects.

Keratoconus: New insights in the underlying pathomechanism by hTNFtg and syndecan-4-deficient mice

U. Hansen*; T. Pap*; N. Eter†; M. Alnawaiseh†

*Institute for Musculoskeletal Medicine, University Hospital Münster, Germany; †Department of Ophthalmology, University of Münster Medical Center, Münster, Germany

Introduction: The corneal stroma consists of orthogonally stacked lamellae formed by thin collagen fibrils evenly spaced in parallel. Among other requirements, activity of lysyl oxidase (LOX) and tissue transglutaminase (TG2) is essential for lamellar stacking and thin fibrils respectively. However, the mechanism for development of this suprastructure is still unknown. Recent studies suggest that loss of syndecan-4 has regulatory effects on cross-linking enzymes, and thereby, resulting in structural ECM changes. Interestingly, in the human eye disease keratoconus (KC), structural and compositional changes leads to disruptions of the lamellar organization with thinning and scarring of the central part of the cornea but pathomechanisms are unknown. Both genetic and environmental factors have been associated with KC, and recent studies suggest that at least in part, inflammatory conditions (eg, high TNF-a levels) might play a role in KC.

Materials and Methods: We have studied the suprastructural organization of the corneal stroma of hTNFtg and syndecan-4-deficient mice as well as of KC patients by TEM. Disruptions of collagen structures were visualized by collagen hybridizing peptides B-CHP. Moreover, 3D-cell cultures of isolated keratocytes were analyzed by TEM and for TG-activity.

Results: Sheets of orthogonally arranged collagen fibrils were found in the stroma of wild-type mice and of human controls. However, lamellae were disrupted in hTNFtg and in syndecan-4-deficient mice. Interestingly, however, a similar morphology of the stroma was found in KC patients. Moreover, keratocytes revealed evidences for apoptosis and collagen fibrils formed were thicker with a visible banding pattern. 3D-cell cultures of human KC keratocytes generated an altered ECM with reduced TG-activity in comparison with controls. Moreover, binding of B-CHP was stronger in KC samples as well as in hTNFtg mice.

Discussion: The disruption of the lamellar organization of collagen fibrils in hTNFtg and syndecan-4-deficient mice is similar to that found in corneal stroma of KC-patients. Thus, cross-linking as well as inflammatory factors could be crucial factors for manifestation of KC supported by more degraded collagens visualized by a higher binding of B-CHP. On the other hand, analysis of syndecan-4-deficient corneas will provide new insights in mechanisms of lamellae formation and fibril diameter control respectively.

Cartilage-specific ERp57 knockout mice qualify as a novel model for the analysis of ER stress-related skeletal diseases

Y. Knieper*; I. Gronau*; U. Hansen†; M. Ramroth*; R. Dreier*

*Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, Germany; †Institute of Experimental Musculoskeletal Medicine, University Hospital Münster, Germany

Introduction: Cartilage is essential for skeletal development by endochondral ossification. The chondrocytes produce extracellular matrix (ECM) proteins that undergo folding in the endoplasmic reticulum (ER). ERp57 is essential as it is involved in disulfide bond formation of ECM glycoproteins. If this process fails, ER stress arises and the unfolded protein response (UPR) is induced. ER stress and the UPR in chondrocytes are implicated in the pathogenesis of chondrodysplasias. We generated a cartilage-specific ERp57 knockout mouse (cKO) and analyzed the effects of ER stress during bone development. Additional *in vitro* analyses in C28I2 cells with CRISPR/Cas9-induced KO of ERp57 (C28/I2-KO) were performed.

Materials and Methods: In WT and cKO littermates, bone characteristics (length, structure, growth plate zones, proliferation, apoptosis) were determined by μCT, (immuno) histochemical and TUNEL staining. Dilated ER cisternae and autophagy were evaluated by TEM. ER stress (Bip, XBP1s, Chop, ATF6, IRE-1) and autophagy marker (LCIII)

expression, as well as metabolic activity of chondrocytes (MTT) were investigated after cultivation with ER stress inductor thapsigargin (Tg) and/or the chemical chaperone 4-phenylbutyrate (4-PBA).

Results: Loss of ERp57 triggered ER stress in growth plate chondrocytes of cKO mice and in C28/I2-KO cells. Accumulation of ECM proteins induced the dilation of ER cisternae, reduced proliferation, accelerated apoptosis and resulted in a chondrodysplasia phenotype with shortened long bones. In C28/I2-KO cells, ER stress reduced the metabolic activity and induced autophagy. Increased levels of ER stress marker proteins were detected in WT and KO cells in presence of 0,0001-10 μM Thapsigargin. However, 10-50 mM 4-PBA downregulated all ER-stress and autophagy marker proteins investigated.

Discussion: We demonstrated that cartilage-specific loss of ERp57 in mice is sufficient to induce ER stress in chondrocytes resulting in a chondrodysplasia phenotype. This mouse model qualifies to study the role of ER stress and beneficial effects of chemical chaperones, such as 4-PBA, also in degenerative cartilage diseases.

Vascular calcification during chronic kidney disease: role of the RAGE/Cathepsin S/elastin peptides axis

A. Wahart*; J. Ortillon*; K. Belmokhtar*; C. Schmelzer†; A. Heinz†; F. Velard‡; C. Boulagnon-Rombi*; C. Terryn*; B. Romier-Crouzet*; H. El Btaouri*; S. Blaise*; P. Maurice*; A. Bennasroune*; P. Gillery*; L. Debelle*; P. Rieu*; F. Touré*; L. Duca*

*UMR CNRS/URCA 7369 MEDyC, Université de Reims Champagne-Ardenne; †Faculty of Natural Sciences I, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany; ‡EA 4691 BIOS, SFR-CAP Santé (FED 4231), Université de Reims Champagne-Ardenne

Introduction: Vascular calcification is a common feature of patients with chronic kidney disease (CKD). We recently reported a role for the Receptor for Advanced Glycation End products (RAGE) in the uremic vascular calcification process following engagement by uremic toxins. Moreover, several studies have suggested the involvement of Cathepsin S in elastolysis and vascular calcification during CKD. However, the link between these actors and the mechanisms implicated and the putative role of RAGE in Cathepsin S expression and the subsequent elastolysis are unknown.

Materials and Methods: We used a mouse model of uremic vasculopathy in the $ApoE^{-/-}$ or $ApoE^{-/-}/RAGE^{-/-}$ (DKO) backgrounds as well as primary cultures of VSMCs isolated from C57Bl6J mice.

Results: We found that induction of CKD increases the calcifications processes in the cardiac valves of $ApoE^{-/-}$ mice whereas DKO are protected. Moreover, aortas analysis showed that Cathepsin S expression and elastolysis seem to be greater in $ApoE^{-/-}$ than in DKO animals. Using recombinant Cathepsin S, we showed by using electron microscopy scanning and mass spectrometry analysis that this protease degrade insoluble elastin producing bioactive elastin-derived peptides (EDPs). Then, we showed that *in vitro* calcification process is triggered when VSMCs are incubated with inorganic phosphate and is increased in the presence of EDPs. At last, the use of an inhibitor of the Elastin Receptor Complex (ERC), DANA, abolished this phenomenon.

Discussion: In conclusion, during CKD, activation RAGE by uremic toxins leads to Cathepsin S expression promoting elastolysis leading to the production of bioactive EDPs. These peptides accelerate vascular calcification by binding on ERC. This study highlights the potential for targeting the ERC to modulate vascular calcification during CKD.

Hyaluronan-binding protein involved in hyaluronan depolymerization (HYBID, alias KIAA1199 or CEMIP) is up-regulated and involved in hyaluronan (HA) degradation in human osteoarthritic cartilage

Y. Okada*,†; H. Shimizu‡; M. Shimoda†; S. Mochizuki†; Y. Miyamae†; H. Abe†; M. Chijiiwa†; H. Yoshida§; J. Shiozawa¶; M. Ishijima¶; K. Kaneko¶; A. Kanaji‡; M. Nakamura‡; Y. Toyama‡

*Department of Pathophysiology for Locomotive and Neoplastic Diseases, Juntendo University Graduate School of Medicine; †Department of Pathology, Keio University School of Medicine; ‡Department of Orthopaedic Surgery, Keio University School of Medicine; §Biological Science Research, Kao Corporation; ¶Department of Medicine for Orthopaedics and Motor Organ, Juntendo University Graduate School of Medicine

Introduction: We showed that HYBID, also called KIAA1199 or CEMIP, plays a key role in degradation of HA in skin and arthritic synovial fibroblasts (PNAS 110:5612-5617, 2013), but its functions in osteoarthritic (OA) cartilage remain elusive. Here, we investigated the expression and roles of HYBID in human OA cartilage.

Materials and Methods: Normal control and OA cartilage samples were obtained from hip joints of the patients with femoral neck fracture and knee and hip joints of OA patients respectively. They were histologically and immunohistochemically examined. mRNA and protein expression of HYBID was studied by real-time PCR and immunoblotting. Chondrocytes isolated from OA cartilage were stimulated with inflammatory cytokines and growth factors and degrading activity of high molecular weight HA was examined by size-exclusion chromatography. Cellular localization of

HYBID and exogenously added HA was observed by confocal microscopy.

Results: HYBID was highly expressed by chondrocytes in the HA-depleted area of OA cartilage, and the HYBID immunoreactivity directly correlated with Mankin score, the histopathological severity of OA lesions of cartilage. Quantitative real-time PCR indicated that the HYBID expression was significantly higher in the OA cartilage than in the control cartilage. In addition, OA chondrocytes exhibited HA-degrading activity, which was abolished by knockdown of HYBID by small interfering RNAs. Although OA chondrocytes also expressed certain levels of hyaluronidase-1, hyaluronidase-2 and CD44, knockdown of these molecules exhibited negligible effects on HA degradation. Double immunostaining of HYBID and clathrin heavy chain revealed that HYBID was localized in the clathrin-coated vesicles and HA was endocytosed within the vesicles in OA chondrocytes. Among eight factors including cytokines and growth factors examined, only tumor necrosis factor-α (TNF-α) stimulated OA chondrocytes to overexpress HYBID.

Discussion: These data are, to the best of our knowledge, the first to demonstrate that HYBID is up-regulated in OA cartilage, and suggest that TNF-α-stimulated HYBID plays a role in HA degradation in OA cartilage.

Understanding skeletal disease with cross-species transcriptomics meta-analysis

J. Soul*; T. Hardingham†; R. Boot-Handford†; J.-M. Schwartz*

*Division of Evolution & Genomic Sciences, Faculty of Biology, Medicine and Health, University of Manchester; †Wellcome Centre for Cell-Matrix Research, Faculty of Biology, Medicine and Health, University of Manchester

Introduction: Skeletal diseases are prevalent in society with a large socio-economic cost and improved molecular understanding is required to develop improved treatments. Results from an increasing number of skeletal transcriptomics experiments provide a new opportunity for mechanistic insight into both fundamental skeletal biology and skeletal disease. However, methods for combined analysis of multiple datasets in a focused disease area are lacking. To address this, we have developed a publicly available online exploration portal, SkeletalVis, for the analysis of skeletal gene expression experiments. This portal will allow exploration and mining across multiple experiments. The integrated analysis of the data will detect similarities between new and existing datasets and identify common features between diseases and experimental models and shared pathogenic mechanisms.

Materials and Methods: A Galaxy pipeline was used to analyse 260 published and available skeletal transcriptomic experiments containing 676 expression responses from ArrayExpress and ENA/SRA in a high-throughput manner to give differentially expressed genes and downstream analysis using standard bioinformatics approaches. The resulting data are available online in an interactive exploration and meta-analysis data portal: http://phenome.manchester.ac.uk/

Results: Analysis of the 676 expression response profiles yielded differential expression and downstream analysis include pathways, sub-networks, drug response and transcription factor enrichment. Using related experiments we demonstrate we can recover prior biological knowledge and identify similar experiments with shared biological mechanisms. Using this data portal, we identify similarities in expression response in a cross-species group of osteoarthritis animal model expression responses. We further compare recently available osteoarthritis expression data to animal and explant tissue models revealing shared differential gene expression and dysregulated matrix signalling pathways between these models and human diseased tissue.

Discussion: The SkeletalVis provides an online openly accessible data portal for exploration and comparison of skeletal transcriptomic data. We demonstrate its utility in identifying both known and novel relationships between skeletal expression signatures. Importantly, SkeletalVis enables users to upload and analyse data from new experiments and to incorporate them in larger scale meta-analysis. Continued expansion of the repository of data sets is planned as published results become available, which will progressively increase its scale and value as a research community resource.

New Zebrafish models for recessive osteogenesis imperfecta

F. Tonelli*; L. Leoni*; S. Cotti*; I. Fiedler†; B. Busse†; E. Witten‡; R. Besio*; G. Giannini*; A. Rossi*; A. Forlino*

*Department of Molecular Medicine, University of Pavia, Pavia, Italy; †Department of Osteology and Biomechanics, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; ‡Department of Biology, Ghent University, Gent, Belgium

Introduction: Osteogenesis imperfecta (OI) is a heritable disorder characterized by bone deformity, skeletal fragility and short stature. Cartilage associated protein (CRTAP), proline 3-Hydroxylase 1 (P3H1) and Cyclophilin B (PPIB) are components of the ER-resident complex, involved in the hydroxylation of specific proline residues in collagen type I

α chains. Mutations in these proteins are responsible for recessive OI type VII, VIII and IX respectively. Murine models for these diseases exist, but the availability of zebrafish models will allow a deeper understanding of the phenotype at early developmental stage as well as will favour drug screening with multiple molecules reducing amount, timing and cost. We applied CRISPR/Cas9 editing strategy to generate knockout models for Crtap and P3 h1 and performed their characterization.

Materials and Methods: Syntheny analysis was performed using Genomycus v87.01. CHOPCHOP was used for guide RNA (gRNA) selection. pT7gRNA vector was used for gRNA subcloning and *in vitro* transcription and the pT3TS-nCas9n plasmid for Cas9 mRNA synthesis. Morphometric measurements of mutants were performed using Leica LAS v4.5 software. Alizarin red staining and x-Rays were used for bone characterization. Mutant collagen type I was characterized by SDS-PAGE. The growth rate of caudal fin was performed measuring length of fin rays after amputation.

Results: *p3 h*1 and *crtap* are highly conserved between tetrapods and teleosts. We successfully generated the knockout for both genes in zebrafish using CRISPR/Cas9. P3 h1 and Crtap homozygous mutants are smaller than WT and show a delayed mineralisation as revealed by alizarin red staining. Their phenotype is worsening with age and adult mutant fish are characterized by vertebrae disorganization and skeletal deformity, as observed by x-Rays and μCT scans. Collagen type I has abnormal electrophoretic migration. Bone formation, evaluated on fin regeneration, was delayed in *p3 h1* mutants compared to WT.

Discussion: We proved the goodness of zebrafish model to reproduce the phenotype of the recessive OI type VII and VIII. Our goal will be to use our models for drug screening in order to pave the way to new pharmacological treatments.

Ablation of epidermal collagen chaperoning by Hsp47 results in dermal fibrosis

A. Köhler*,†; M. Mörgelin‡; J. Brinckmann§; M. Aumailley*; F. Zaucke¶; U. Baumann†; G. Sengle*,**

*Center for Biochemistry, Medical Faculty, University of Cologne; †Institute of Biochemistry, Faculty of Mathematics and Natural Sciences, University of Cologne; ‡Department of Clinical Sciences, Division of Infection Medicine, Lund University; §Institute of Virology and Cell Biology, University of Lübeck ; ¶Dr Rolf M Schwiete Research Unit for Osteoarthritis, Orthopedic University Hospital, Friedrichsheim GmbH, Frankfurt/Main; **Center for Molecular Medicine Cologne, University of Cologne

Introduction: Heat-shock protein 47 (Hsp47), an essential chaperone during procollagen biosynthesis, is responsible for the stabilization of pro-collagen and its secretion into the extracellular matrix. Mutational inactivation in humans or genetic ablation in mice showed that Hsp47 is indispensable for bone and cartilage formation during development, but also specific ablation in the dermis leads to embryonic lethality. Hsp47 is described to principally interact with fibrillar collagens; however, it is not fully investigated whether also other members of the collagen superfamily such as transmembrane collagens require Hsp47 assistance for triple-helix stabilization and proper network assembly. To explore a role of Hsp47 on epidermal transmembrane collagen chaperoning *in vivo* we generated epidermal specific Hsp47 knockout (Hsp47 eKO) mice.

Materials and Methods: K14 Cre/loxP system; electron microscopy; second harmonic generation microscopy; mass-spectrometry; Western blot analysis; collagen-crosslink analysis; confocal immunofluorescence microscopy; and ELISA-style binding assay.

Results: The keratinocyte-specific genetic ablation of Hsp47 in mice results in severe dermal fibrosis characterized by altered dermal collagen crosslinking and fibril formation. Isolated Hsp47-deficient basal keratinocytes revealed significant reduction of collagen XVII expression and shedding which was accompanied by increased secretion of TGF-β. ELISA-style binding assays identified collagen XVII as new interaction partner for Hsp47.

Discussion: Our data show that Hsp47 interacts with members of the MACIT (membrane-associated collagens with interrupted triple helices) collagen subfamily such as collagen XVII. Our finding that epidermal ablation of Hsp47 leads to excessive dermal fibrosis suggests a so far unknown role of Hsp47 in epidermal-dermal cross-talk.

Live optical super-resolution microscopy applied to the characterization of maturation, localization and trafficking of defective dystroglycan mutants

W. Hübner*; M. Bozzi†,‡; A. Kerbs*; V. Mönkemöller*; F. Sciandra‡; M. G. Bigotti§; A. Brancaccio‡,§; T. Huser*

*Biomolecular Photonics, Dept. of Physics, Bielefeld University, 33615 Bielefeld, Germany; †Istituto di Biochimica e Biochimica Clinica, Università Cattolica del Sacro Cuore, Roma, Italia; ‡Istituto di Chimica del Riconoscimento Molecolare (CNR), Roma, Italia; §School of Biochemistry, University of Bristol, Bristol, United Kingdom

Introduction: The ubiquitous dystroglycan (DG) adhesion complex is located at the plasma membrane where it provides an essential link between the extracellular matrix and the cytoskeleton. Dystroglycan is expressed as a pro-peptide which undergoes a series of maturation steps whereby the precursor is cleaved into an a (extracellular) and b (transmembrane) subunit and heavily glycosylated. Dysfunctional dystroglycan complexes are linked to several autosomal recessive neuromuscular disorders

such as severe congenital (muscle-eye-brain and Walker-Warburg syndrome) or limb-girdle muscular dystrophies (LGMD2P). Moreover, genetic abnormalities of glycosyltransferases leading to hypoglycosylated a-DG result in a reduced affinity towards the extracellular matrix protein laminin-2. Primary dystroglycanopathies are those caused by direct missense mutations of the dystroglycan core protein, and we are establishing models for studying them with a multidisciplinary approach.

Materials and Methods: We use a combination of several fluorescence microscopy techniques, from advanced to super-resolution fluorescence microscopy, such as 3D structured illumination. For that purpose, the dystroglycan subunits are individually fluorescently labeled. Wild-type DG expression is compared to site-directed missense mutations in live cells.

Results: Live cell super-resolution microscopy allows us to efficiently and reliably track DG's subcellular localization. Trafficking of the dystroglycan complex towards the plasma membrane during processing, as well as some retrograde movements, is observed.

Discussion: This detailed imaging approach illustrates the severe molecular and trafficking defects in dystroglycan mutations involved in LGMD2P and muscle-eye-brain disease. We believe that our experimental work can be crucial to understand the complex maturation pathway of dystroglycan and its subunits within the cell.

Structural model of domain 1 of the a-dystroglycan glycosylating enzyme LARGE

A. Brancaccio*,†; B. Righino‡; M. Bozzi*,‡; F. Sciandra*; M. G. Bigotti†; D. Pirolli*; M. C. De Rosa*

Istituto di Chimica del Riconoscimento Molecolare (CNR), Roma, Italia; †School of Biochemistry, University of Bristol, Bristol, United Kingdom; ‡Istituto di Biochimica e Biochimica Clinica, Università Cattolica del Sacro Cuore, Roma, Italia

Introduction: Dystroglycanopathies are characterised by hypoglycosylation of a-dystroglycan (DG) that affects binding to extracellular matrix molecules both in muscle and brain. Dystroglycanopathies depending on an aberrant O-glycosylation of a-DG are associated with several genes, including POMT1/2, FKTN, FKRP, POMGNT2 and LARGE. LARGE is a two-domain glycosyltransferase whose function is to add multiple repetitions of a disaccharide responsible for laminin binding. The structural features of LARGE and how it interacts with a-DG remain still unknown. Here, we present the first structural model of domain 1 of LARGE that we also used as a structural basis to evaluate the effects of a missense mutation (S311F) resulting in congenital muscular dystrophy with brain and eye anomalies.

Materials and Methods: Two fold-recognition algorithms (I-TASSER and HHPRED) have been employed to build the three-dimensional structure of LARGE domain 1. The Build Mutant protocol implemented in Discovery Studio (Biovia) was used to generate a structural model of the S331F mutant. The stability of the wild-type protein and the effects of the mutation have been further investigated by means of molecular dynamics (MD) simulations using the program Desmond.

Results: The first-ranking structures from the two fold-recognition algorithms indicate the same fold, corresponding to the one of galactosyltransferase LgtC. The S311F replacement results in an increase of hydrophobicity in the vicinity of the catalytic site, and MD simulations clearly point out an altered flexibility of the backbone of the enzyme upon mutation. The model has also been used to set up a preliminary recombinant expression system of domain 1.

Discussion: We employed molecular modelling studies to generate a reliable model structure of the domain 1 of LARGE. The model represents the structural basis for computational mutagenesis studies, for model building of the entire protein and for protein-protein docking with α-DG.

Implications of Gas1 in Synovial Joint Formation and Disease Mechanism of Brachydactyly Type A1

A. Leung; C. Feng; X. Weng; D. Chan

School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China

Introduction: Brachydactyly type A1 (BDA1) is a congenital disease causing reduced digit lengths and a missing joint in digit V, resulting from homozygous E95K mutation in Ihh gene. IhhE95K has expanded signaling field, thus higher Ihh concentration in developing joint. Preliminary studies show initiation but incomplete development of joint primordia leading to a missing joint, concomitant with decrease in cell death. Growth arrest specific-1 (Gas1) is a joint expressing HH binding protein shown to have dependency receptor characteristics in the absence of HH inducing apoptosis. Gas1 is expressed in the developing joint. We hypothesize that increased level of IHH in BDA1 joints reducing cell death as a development mechanism for the missing joint.

Materials and Methods: Joint development in BDA1 mouse embryos was systematically analysed for differentiation and cell death markers in relation to Gas1. *Ex vivo* joint cultures are subjected to treatment with general caspase inhibitor zFAD-FMK, to assess the significance of apoptosis in joint formation. To understand the role of Gas, a conditional mouse for an activation of Gas1 was generated.

Results: We confirmed all phalangeal interzones are initiated in BDA1 mice. However, some distal interzones in BDA1 mice failed to progress and a joint is not formed, causing brachydactyly. TUNEL signals are detected in the centre of Gas1 expressing digit interzones associated with the cavitation event, correlating with development progression of the individual joints. Cleaved caspase-3 is detected but much less in comparison with TUNEL. However, *ex vivo* joint cultures treated with zFAD-FMK, an apoptosis inhibitor did not prevent joint cavitation in wt mice, suggesting alternative modes of cell death, such as necroptosis. In BDA1, despite Gas1 expression remains in interzone, TUNEL is reduced that is not significant in distal joints, consistent with the phenotype.
Discussion: A relationship between cell death, joint cavitation and BDA1 phenotype was demonstrated. Whether cell death is associated with canonical apoptotic pathways needs to be further studied. The potential of Gas1 in regulating the cell death event and cavitation in joint formation will be tested as we have generated a conditional mouse for an activation of Gas1 and the level of Ihh in disease mechanism of BDA1.

Extracellular matrix disarray and αvβ3 integrin-ILK-Snail1-mediated signaling are involved in a fibroblast-to-myofibroblast transition in hypermobile Ehlers-Danlos syndrome

N. Zoppi; N. Chiarelli; S. Binetti; G. Carini; M. Ritelli; M. Colombi

Department of Molecular and Translational Medicine, Division of Biology and Genetics, University of Brescia, Brescia, Italy

Introduction: During tissue injury, fibroblasts differentiate to myofibroblasts regulating synthesis, remodeling, reabsorption of the extracellular matrix (ECM), and inflammation's resolution. The organization of the α-smooth muscle actin (α-SMA) cytoskeleton allows myofibroblasts' migration and ECM contraction. The persistent myofibroblasts' activity contributes to chronic inflammation.
Ehlers-Danlos syndromes (EDS) are a heterogeneous group of heritable connective tissue disorders sharing skin hyperextensibility, joint hypermobility (JHM), and tissue fragility. Classical EDS (cEDS), caused by defects in type V collagen (COLLV), is characterized by marked cutaneous involvement and generalized JHM. Vascular EDS (vEDS), due to COLLIII defects, is characterized by arterial rupture/dissection/aneurysm and organ ruptures. Hypermobile EDS (hEDS), orphan of a genetic etiology, is primarily identified as having generalized JHM, related musculoskeletal manifestations, and a mild skin involvement. The hEDS-associated phenotypic spectrum is broad and includes multiple signs and symptoms shared with chronic inflammatory systemic diseases. To shed light into pathomechanisms underlying hEDS, we performed gene expression profiling of hEDS dermal fibroblasts together with a detailed cellular characterization of these cells in comparison with those of cEDS and vEDS.
Materials and Methods: Transcriptome-wide expression profiling was carried out by microarray technology. ECM and integrins organization, α-SMA-cytoskeleton, inflammatory markers, and the integrin-mediated transduction pathway were investigated by immunofluorescence microscopy and Western blotting. The migratory capability was measured by the Transwell migration assay.
Results: The hEDS cells showed significant expression changes of several genes involved in maintenance of ECM organization/homeostasis and in immune, inflammatory, and pain responses. These cells shared with cEDS and vEDS fibroblasts the disassembly of COLLs-, fibrillins-, fibronectin (FN)-ECM and the organization of the αvβ3 integrin. Only in hEDS cells, the ECM disarray was associated with increased levels of the metalloproteinase-9, presence of proteolytic FN-fragments in cells' media, organization of the α-SMA-cytoskeleton, enhanced migration, altered expression of the inflammation mediators CCN1 and CTGF, and an ILK-mediated αvβ3 integrin signaling involving the transcription factor Snail1, consistent with a fibroblast-to-myofibroblast transition.
Discussion: The myofibroblast-like phenotype distinguishes hEDS from cEDS and vEDS fibroblasts and suggests an *in vitro* inflammatory-like condition, which correlates well with the systemic clinical manifestations of the patients.

Investigating the tumour ECM in the inhibition of anti-tumour immunity

E. Puttock*; J. Rastrick†; A. White†; O. M. T Pearce*

**Centre for Cancer Inflammation, Barts Cancer Institute, QMUL, Charterhouse Square, London, EC1M 6BQ; †UCB Pharma, Slough, SL1 3WE*

Introduction: Extracellular matrix (ECM) degradation and remodelling occurs in invasive malignant progression, resulting in the expression of unusual matrix proteins in the tumour microenvironment (TME). Whether the remodelled ECM hinders or promotes tumour progression remains controversial. In our work, we identified a tumour matrix index (MI) that correlates with an immunosuppressive phenotype that is conductive of tumour progression. In this work we aim to investigate the direct effect of tumour ECM on immune cell phenotype.
Materials and Methods: HGSOC patient tissue is decellularized to generate an *in vitro* 3-D tissue platform for immune

cell co-cultures using human macrophages, later work will focus on other immune cells. Decellularized tissue is characterised by matrisome proteomics, IHC, H&E and assigned a disease score using Definiens® digital image analysis. Flow cytometry using a panel of macrophage antibody markers, qPCR and IHC is used to characterize macrophage phenotype. Proteomics informed by transcriptomics (PIT) is used to identify tumour specific MI protein isoforms expressed in decellularized tissues.

Results: Decellularized tissues maintain ECM integrity and the MI. Monocytes or macrophages can be incubated with decellularized tissues, and later isolated from the tissue for flow or qPCR analysis. Direct macrophage-decellularized tissue interaction can be assessed by IHC and IF.

Discussion: Decellularized human tissue provides a platform to study immune cell-ECM interactions. From our data so far, matured macrophages are viable for over 9 days and can be phenotyped after co-culture end points. We are currently determining how diseased tissue influences macrophage phenotype. This work may also be useful for other diseases associated with fibrosis.

Human rhinovirus infection of airway epithelial cells induces tenascin-C release

J. Mills*; I. Sabroe*; K. Midwood†; L. Parker*

*Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield;; †Kennedy Institute of Rheumatology, University of Oxford

Introduction: Viral infections are the cause of 75% of all asthma exacerbations, with human rhinovirus (HRV) being the most common. Tenascin-C (TNC) is an ECM protein that is present in small quantities in the airway of healthy individuals, but in high quantities in asthma sufferers; however, the inflammatory potential of TNC in asthma has yet to be investigated. We hypothesise that HRV infection induces the upregulation of TNC expression in the airway, contributing to increased inflammatory cytokine production.

Materials and Methods: C57BL/6 mice were intranasally administered with the viral mimic Poly(I:C) and bronchoalveolar lavage fluid (BALF) analysed for TN-C. BEAS-2B cell line and primary human bronchial epithelial cells (PBECs) were stimulated with Poly(I:C) or infected with HRV and assayed for TN-C mRNA, protein expression and release. Finally, recombinant TN-C was added exogenously to BEAS-2B cells and extracellular vesicles (EVs) were isolated following stimulation, analysed for TN-C expression and used to stimulate BEAS-2B cells and macrophages.

Results: TNC expression in the mice BALF was significantly upregulated following Poly(I:C) stimulation, and *in vitro* Poly(I:C) and HRV treatment induced TN-C mRNA and TN-C release in PBECs and BEAS-2B cells, highlighting a novel relationship between HRV infection and airway TN-C expression. Viral-induced TN-C release was significantly higher from asthmatic PBECs compared to non-asthmatic PBECs, demonstrating an increased prominence in a disease setting. Furthermore, Poly(I:C) stimulation also induced EV release and EV-associated TNC expression in BEAS-2B cells. EVs derived from Poly(I:C) stimulation induced cytokine release from BEAS-2B cells and macrophages, as did stimulation with recombinant TN-C, demonstrating the inflammatory potential of EVs and TN-C in the airway following viral treatment.

Discussion: Further work to determine the effects of TNC on inflammation in the airway is ongoing. The current data support a potential link between TNC release following HRV infection and inflammatory cytokine production in the airway, potentially through the release of EVs.

TSG-6 modulates chondrocyte phenotype in osteoarthritis by suppressing inflammatory signals that promote cartilage matrix breakdown

S. P. Drummond*; S. Anand‡; L. C. Biant†,§; A. J. Day*; C. M. Milner†

*Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, United Kingdom; †Faculty of Biology, Medicine & Health, University of Manchester, United Kingdom; ‡Department of Orthopaedics, Stepping Hill Hospital, Stockport, United Kingdom; §Manchester Orthopaedic Centre, Trafford General Hospital, Manchester, United Kingdom

Introduction: TSG-6 is a secreted protein expressed during inflammation that has diverse tissue-protective and anti-inflammatory activities. TSG-6's ability to modulate the interactions of matrix molecules with cell surface receptors (eg, HA/CD44 binding) and with immune regulators (eg, HSPG/chemokine binding) likely underpins many of its beneficial properties. Here, we investigated the role of TSG-6 as an inhibitor of cartilage damage in the context of osteoarthritis (OA).

Materials and Methods: Human chondrocytes, derived from bone marrow stem cells, were cultured as 3D pellets in the presence of IL-1b or TNF ± recombinant human (rh) TSG-6 or its isolated Link module domain (Link_TSG6); ADAMTS5 and MMP13 gene expression were determined by quantitative PCR. Cartilage explants from OA patients undergoing knee arthroplasty were cultured in the absence/presence of IL-1b/oncostatin-M ± rhTSG-6 or Link_TSG6; glycosaminoglycan loss was quantified. RNAscope probes were used to quantitate expression of TSG-6, ADAMTS5 and MMP13 mRNAs by chondrocytes in OA cartilage.

Results: Link_TSG6 and rhTSG-6 suppressed cytokine-induced gene expression of the proteases MMP13 and ADAMTS5 (key mediators of cartilage breakdown in OA) by cultured chondrocytes, where Link_TSG6 was substantially more potent than the full-length protein. Consistent with this, the loss of proteoglycans from human OA cartilage explants was significantly inhibited by Link_TSG6 treatment. RNAscope experiments revealed a negative correlation between TSG-6 and MMP13 expression by chondrocytes in the superficial regions of damaged OA cartilage; however, we saw a positive correlation between the expression of TSG-6 and ADAMTS5.

Discussion: Our data reveal that TSG-6 can modulate chondrocyte phenotype by suppressing cellular responses to inflammatory signals, thereby reducing OA-associated cartilage damage. We are now investigating potential mechanisms for TSG-6's inhibition of MMP13 expression and for its effects on ADAMTS5 activity, which might involve regulation of both cytokine-induced expression and receptor-mediated endocytosis. TSG-6 has been shown in other systems to mediate anti-inflammatory effects in a CD44-dependent manner, such that TSG-6's enhancement of CD44/HA interactions might represent a mechanism for its regulation of chondrocyte phenotype. Alongside this, we are developing Link_TSG6 as a biological to treat OA by harnessing the intrinsic protective properties of TSG-6 in cartilage.

Leukocyte migration and inflammatory disease: the collaboration between endothelial sugars and chemokines

D. P. Dyer[*,†]; C. L. Salanga[*]; E. Migliorini[‡,§]; D. Thakar[§]; T. Kawamura[*]; G. Graham[†]; R. P. Richter[‡,§,¶]; T. M. Handel[*]

[*]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, California 92093-0684; [†]Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, G12 8TA, United Kingdom; [‡]CIC biomaGUNE, 20009 Donostia-San Sebastian, Spain; [§]Département de Chimie Moléculaire, Université Grenoble Alpes - CNRS, 38041 Grenoble Cedex 9, France; [¶]School of Biomedical Sciences and School of Physics and Astronomy, University of Leeds, Leeds, LS2 9JT, United Kingdom

Introduction: Chemoattractant cytokines (chemokines) are integral in recruitment of leukocytes to inflammatory sites and associated inflammatory pathologies, for example, autoimmune diseases. Chemokine function is dependent on their ability to bind to, and be presented on, cell surface glycosaminoglycans (GAGs), mediating the formation of chemotactic gradients, enabling presentation and signaling through receptors on leukocytes. GAGs are integral extracellular matrix components that not only support chemokine localisation but also help to form the glycocalyx, which acts as a physical barrier to leukocyte adhesion and subsequent migration. Chemokine oligomerization is essential to *in vivo* chemokine-mediated leukocyte recruitment (eg, CCL2 and CCL5); however, monomeric chemokine is largely sufficient for receptor signaling, leaving the mechanistic importance of oligomerization unresolved.

Materials and Methods: We have utilized surface plasmon resonance (SPR) and quartz crystal microbalance with dissipation (QCMD) alongside endothelial binding to examine the interaction of chemokines, and mutants, with different isolated GAGs and the cell surface.

Results: These results demonstrate that the majority of chemokines bind to heparin and heparan sulfate (HS) with a range of affinities, and more selectively with chondroitin sulfate-A. Inhibition of chemokine oligomerization suggests that this process mediates the nature, strength and selectivity of some chemokine-GAG interactions. Our data also suggest that chemokines have different abilities to cross-link individual HS chains on a biosensor. Importantly, this process may provide a novel mechanism that enables retention of chemokines on the cell surface and subsequent modification of the glycocalyx. Chemokine-mediated HS cross-linking also appears to be dependent upon oligomerization, in the case of CCL2, CCL5 and CXCL4. Therefore, there appears to be an overlap between GAG-binding, HS chain cross-linking and chemokine oligomerization

Discussion: We conclude that oligomerization enables binding to, and modification of, GAG chains, enhancing retention on cell surfaces whilst also enabling chemokine-mediated GAG re-organization. This enables chemokine cell-surface localization under flow and thus leukocyte recruitment *in vivo* and potentially suggests a novel function of chemokines in physical re-organization of the glycocalyx.

Leukocyte control of matrix deposition in the lymph node

V. G. Martinez[*]; A. C. Benjamin[*]; V. Pankova[*]; C. J. Tape[†]; S. E. Acton[*]

[*]MRC Laboratory for Molecular Cell Biology, University College London, London, United Kingdom; [†]Cancer Institute, University College London, London, United Kingdom

Introduction: To orchestrate the adaptive immune response, lymph nodes (LNs) require a highly organised tissue architecture supported by stromal cell populations. Fibroblastic reticular cells (FRCs) comprise the most abundant LN stromal cells. FRCs express podoplanin (PDPN), only known endogenous ligand of the C-type lectin receptor CLEC-2. These specialised fibroblasts produce and enwrap the conduit, a tubular network of collagens and

associated proteins that controls intranodal flow of lymphborne factors. LNs expand 3-5 times in size with each immune response, but whether these structures are remodelled during the rapid and reversible expansion of LNs is unknown. During LN expansion, migratory dendritic cells (DCs) expressing CLEC-2 inhibit contractility the FRC network, allowing initial expansion of the LN. Here, we study that this same interaction also regulates remodelling of the extracellular matrix of LNs.

Materials and Methods: Conduit network integrity was studied ex vivo by different histology techniques. Phosphoproteomic and transcriptomic analysis was used to study how CLEC-2 binding to $PDPN^+$ FRCs regulates signalling cascades controlling matrix deposition and expression of matrix components. Functional *in vitro* assays and 3D DC-FRC co-cultures were used to demonstrate mechanisms involved.

Results: Histological analysis shows a marked reduction in collagens within the conduit network in LNs during the immune response. By RNAseq we found that FRCs express > 400 matrisome genes, of which nearly 40% were regulated by CLEC-2/PDPN interactions, including downregulation of Col6a1, Col6a2, Col4a5 and Col4a6. We characterised *in vitro* FRC-derived 3D matrices by immunofluorescence and mass spectrometry, finding that CLEC-2 reduces total matrix deposition and modifies fibre bundling and alignment. Mechanistically, we found that CLEC-2 expressed by DCs disrupts focal adhesion in FRCs affecting microtubule attachment to the membrane, essential for localized matrix deposition.

Discussion: Our data demonstrate a novel mechanism by which DCs regulate expression and deposition of extracellular components by fibroblasts. This mechanism seems to act regulating the conduit network in draining LNs after immunization. Loss of fibrillar components may reduce organ stiffness and therefore facilitate rapid LN expansion. It is therefore likely that function of the conduit network will be compromised during the onset of the immune response.

Spontaneous atopic dermatitis due to immune dysregulation in mice lacking Adamts2 and 14

L. Dupont*; G. Ehx†; M. Chantry*; C. Monseur*; C. Leduc*; L. Janssen*; D. Cataldo‡; M. Thiry§; C. Jerome¶; J.-M. Thomassin¶; B. Nusgens*; J. Dubail*,**; F. Baron†; A. Colige*

*The Laboratories of Connective Tissues Biology; †The Laboratories of Hematology, ; ‡Biology of Tumor and Development; §Cell Biology; ¶CERM, University of Liege, Belgium; **Department of Genetics, INSERM UMR1163, Institut Imagine, Paris, France*

Introduction: Since its first description, ADAMTS14 has been considered as an aminoprocollagen peptidase based on its high similarity with ADAMTS3 and ADAMTS2.

Materials and Methods: Since the importance of ADAMTS14 for procollagen processing was never experimentally demonstrated *in vivo*, we generated *Adamts14*-deficient mice.

Results: These mice are healthy, fertile and display normal aminoprocollagen processing. They were further crossed with *Adamts2*-deficient mice to evaluate potential functional redundancies between these two highly related enzymes. Initial characterizations made on young *Adamts2-Adamts14*-deficient animals showed the same phenotype as that of *Adamts2*-deficient mice, with no further reduction of procollagen processing and no significant aggravation of the structural alterations of collagen fibrils. However, when evaluated at older age, *Adamts2-Adamts14*-deficient mice surprisingly displayed epidermal lesions, appearing in 2-month-old males and later in some females. Immunohistological evaluations of skin sections around the lesions revealed thickening of the epidermis, hypercellularity in the dermis and extensive infiltration by immune cells. Additional investigations, performed on young mice before the formation of the initial lesions, revealed that the primary cause of the phenotype was not related to alterations of the epidermal barrier but was rather the result of an abnormal activation and differentiation of T lymphocytes towards a Th1 profile. However, the primary molecular defect probably does not reside in the immune system itself since irradiated *Adamts2-Adamts14*-deficient mice grafted with WT immune cells still developed lesions.

Discussion: While originally created to better characterize the common and specific functions of ADAMTS2 and ADAMTS14 in extracellular matrix and connective tissues homeostasis, the *Adamts2-Adamts14*-deficient mice revealed an unexpected but significant role of ADAMTS in the regulation of immune system, possibly through a cross-talk involving mesenchymal cells and the TGFβ pathways.

Post-translational modifications of the extracellular matrix: key events in disease pathogenesis

A. Schwenzer

Kennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom

Introduction: The extracellular matrix (ECM) is both complex and dynamic; its composition and structure vary from one tissue to the next, and it is profoundly altered throughout development, as we age, and during disease. One key factor defining ECM content is post-translational modification of its protein constituents. However, surprisingly little is known about how the matrix is modified, and how this impacts ECM function. Citrullination, the conversion of the

amino acid arginine to citrulline, is significantly elevated at sites of inflammation. My work investigates how this post-translational modification of ECM proteins shapes innate and adaptive immune responses, and how these processes contribute to pathological autoimmunity in rheumatoid arthritis (RA).

Materials and Methods: Distinct citrullinated sites on the matrix molecule tenascin-C were defined by mass spectrometry, and the functional consequences of these modifications on immune cell behaviour investigated. Citrullinated tenascin-C provoked elevated innate immune responses, creating a mobile, aggressive macrophage phenotype, by reducing cell adhesion and enhancing release of a unique signature of pro-inflammatory cytokines. Peptide mapping and protein mutagenesis identified the epitope within tenascin-C responsible for these effects. Targeted inhibition of inflammatory signalling pathways revealed a complex interplay between the immune sensor toll-like receptor 4 and macrophage integrins that is essential for the resolution of inflammation, but which citrullinated tenascin-C escapes, due to the loss of key arginines in integrin binding sites.

Results: I also defined different citrullinated sites on tenascin-C that activate adaptive immunity in people with RA. These modified epitopes were capable of breaking immune tolerance, generating citrulline-specific pathogenic autoantibodies that are the hallmark of this disease. Autoantibody responses towards one immunodominant epitope were examined in large patient cohorts and found to be an accurate means by which to diagnose established RA, as well as identifying people at risk of developing disease, years before the detection of clinical symptoms.

Discussion: My data demonstrate that ECM post-translational modification drives aberrant inflammation via multiple mechanisms: through breaking immune tolerance and the formation of pathogenic autoantibodies, and by activating innate pathways in manner that is no longer subject to homeostatic control. Understanding more about matrix modification therefore can provide novel approaches to disease diagnosis and treatment.

CXCL6: a glycosaminoglycan-binding chemokine with pro-inflammatory activities that are modulated by heparin and TSG-6

N. Kouvatsos*,†; R. Dodd*,†; F. Mahmood†; C. M. Milner†; F. Dell'Accio‡; A. J. Day*,†

*Wellcome Trust Centre for Cell-Matrix Research; †Faculty of Biology, Medicine and Health, University of Manchester, Manchester, United Kingdom; ‡Centre for Experimental Medicine and Rheumatology, Queen Mary University of London, United Kingdom

Introduction: CXCL6 plays a role in cartilage homeostasis, where this chemokine may be retained in the chondrocyte pericellular matrix via its interaction with heparan sulphate proteoglycans (HSPGs). In osteoarthritis (OA), the release of CXCL6 from cartilage matrix is thought to reduce chondrocyte viability and has been hypothesised to attract neutrophils, leading to further tissue damage. Here, we compared the interactions of CXCL6 and CXCL8 with glycosaminoglycans and their effects on leukocyte migration. We also investigated whether TSG-6 (a tissue-protective protein made during inflammation) interacts with CXCL6 and how this modulates its function.

Materials and Methods: Plate-based assays and affinity chromatography were used to characterise chemokine-glycosaminoglycan interactions. Chemotaxis and transendothelial migration experiments were done using Transwell assays. WT and mutant Link_TSG6 proteins were expressed in *E. coli* and their interactions with CXCL6 (and other chemokines) were analysed by surface plasmon resonance.

Results: CXCL6 (like CXCL8) interacted with heparin and highly sulphated HS but exhibited essentially no binding to other glycosaminoglycans. In Transwell assays, CXCL6 promoted chemotaxis and transendothelial migration of HL-60 cells (a neutrophil cell-line) and of a pre-B cell-line expressing the CXCR2 receptor. Additionally, soluble heparin/HS inhibited CXCL6 chemotaxis activity while the effect on CXCL8 was minimal. The Link module of human TSG-6 (Link_TSG6) was found to bind to CXCL6, where this interaction inhibited the association of CXCL6 with heparin (as is the case for some other chemokines) but didn't interfere with binding to the CXCR2 receptor. As a result, Link_TSG6 reduced CXCL6-mediated transendothelial migration but not chemotaxis. Additionally, a mutant of Link_TSG6 was identified that had impaired binding to CXCL8 and also abolished Link_TSG6's inhibitory effect on CXCL8-mediated transmigration. This Link_TSG6 mutant also had reduced binding affinity for CCL2 and CXCL6 (known ligand of TSG-6).

Discussion: Here, we have shown that the promotion of chemotaxis by CXCL6 (unlike CXCL8) is inhibited by heparin/HS. These data suggest that degraded HS chains would inhibit CXCL6's pro-inflammatory effect, for example, in OA cartilage. We found that Link_TSG6 (which we are developing as a biologic for OA) also inhibits CXCL6-mediated leukocyte migration and that there may be a common chemokine binding site on TSG-6.

Role of Interleukin-13 in the Extracellular Matrix Turnover during *Nippostrongylus brasiliensis* Infection

S. Rosini*,†; A. L. Chenery*,†; T. E. Sutherland†; S. Pearson†; Q.-J. Meng*,‡; K. E. Kadler*,‡; J. E. Allen*,†

*Wellcome Trust Centre for Cell-Matrix Research, Oxford Road, Manchester, United Kingdom; †Division of Infection, Immunity and Respiratory Medicine, Oxford Road, Manchester, United Kingdom; ‡Division of Cell Matrix Biology and Regenerative Medicine, Oxford Road, Manchester, United Kingdom

Introduction: Fibrosis is an excessive and disorderly accumulation of insoluble collagen fibres as the result of tissue injury, causing a loss of tissue specialised function. Infection with the rodent nematode, *Nippostrongylus brasiliensis* is an established model to evaluate repair of acute lung injury caused by nematode migration through the parenchyma. Lung repair is dependent on the T helper 2 (Th2) cell cytokines interleukin-4 (IL-4) and -13 (IL-13), which interact with the shared IL-4Ra receptor subunit. It has been previously demonstrated that during skin repair, IL-4Ra signalling to macrophages induces expression of Resistin-like molecule alpha (Relm-a), which in turn induces the expression of the collagen cross-linking enzyme lysyl hydroxylase 2 (LH2). The specific role of IL-13 in lung repair has not been investigated yet during *N. brasiliensis* infection.

Materials and Methods: C57BL/6 wild-type and *Il13*$^{-/-}$ mice were infected with 250 infective larvae of *N. brasiliensis*. Lungs and bronchoalveolar lavages (BAL) were harvested 2, 4, and 6 days after infection. Gene expression profile, flow cytometry, immunohistochemistry and ELISA were performed.

Results: IL-13 regulated infiltration of neutrophils and eosinophils in *N. brasiliensis* infection. Relm-a expression in lungs and abundance in BAL was increased upon *N. brasiliensis* infection and was controlled by IL-13. Additionally, the expression of the transmembrane matrix metalloproteinase MMP-14 was regulated by IL-13 in lungs.

Discussion: These data suggested that during injury, IL-13 might regulate the extracellular matrix turnover via Relm-a. This study gives important insights to fully understand IL-13's pro-fibrotic activities and the link between type 2 immunity and initiation of pro-fibrotic pathways.

Up-scale manufacture of chondrocytes in the development of allogeneic cartilage therapies

J. Garcia*,†; C. Mennan*,†; C. Hulme*,†; S. Roberts*,†; R. Banerjee†; C. Meyer†; K. Wright*,†

*Keele University, Institute of Science and Technology in Medicine; †Robert Jones and Agnes Hunt Orthopaedic Hospital NHS Trust

Introduction: There is a requirement for chondrocyte therapy, to become more cost-efficient if it is to reach a wider patient population. An allogeneic product would improve the cost-effectiveness and widespread use of chondrocyte therapy.

Materials and Methods: Macroscopically normal cartilage from three donors undergoing knee arthroplasty was used to derive chondrocyte cultures in media supplemented with human platelet lysate (HPL, Stemulate®) in the Quantum® Cell Expansion System or on tissue culture plastic (TCP) with foetal bovine serum (FBS) or HPL. The Quantum® houses a disposable bioreactor consisting of ~11.5 k fibres, providing a growth surface of ~2.1 m^2. Cell yields and the number of population doublings were calculated. Flow cytometry analysis was used to assess immunoprofiles on freshly isolated, Quantum® and TCP chondrocytes. Chondrogenesis was assessed using standard pellet culture and glycosaminoglycan (GAG) assays.

Results: Chondrocytes grown in FBS cf. HPL had distinct rounded/cuboidal compared to biopolar/fibroblastic morphologies. The Quantum® produced higher cell yields (75 ± 38 M) cf. FBS supplemented (2.6 ± 0.4 M) or HPL supplemented (6.9 ± 3.8 M) TCP cultures. The number of population doublings for Quantum®, TCP FBS and TCP HPL being 2.82 ± 1.18, 1.57 ± 1.1 and 1.3 ± 0.12 respectively. Quantum® chondrocytes displayed similar surface immunoprofiles to those grown on TCP and unlike freshly isolated cells, were immunopositive for CD90, CD73, CD105, CD166, CD151 and all integrins tested. Chondrogenic pellet analysis demonstrated that all cultures produced GAGs to varying degrees.

Discussion: Our preliminary results show that compared to traditional methods, the Quantum® is capable of generating higher numbers of chondrocytes, which demonstrate comparable characteristic immunoprofiles and cartilage forming capacity. The Quantum® has the potential to reduce manufacturing costs for multiple dose allogeneic chondrocyte banking. Further work will examine the effects of Quantum® expansion on gene expression profiles, telomere length and evaluate the cartilage forming capacity of Quantum® products in preclinical models of cartilage injury.

Murine cruciate ligament pathology and mechanics during osteoarthritis development

L. Ramos*; G. Bou-Gharios*; A. A. Pitsillides†; E. Comerford*; B. Poulet*

*Institute of Ageing and Chronic Disease, University of Liverpool, United Kingdom; †Comparative Biomedical Sciences, The Royal Veterinary College, United Kingdom

Introduction: Osteoarthritis (OA) is a multicomponent joint disease characterized by articular cartilage degeneration, but also affecting surrounding tissue. Of interest are the ligaments which have been closely linked to OA in humans and in OA animal models. Our goal was to study the pathological changes in the ligaments during disease progression in murine spontaneous and posttraumatic OA.

Materials and Methods: Histological sections were taken from three different OA models: STR/ort mice, CBA mice

following non-invasive knee trauma3, and C57Bl/6 mice following DMM surgery. Samples were imaged for μCT and stained with toluidine blue. Immunohistochemistry was performed at different OA progression stages; markers included cartilage matrix (collagen type II, and sox9), ligament markers (SCXA) and small leucine-rich proteoglycans (asporin). Gait analysis was analysed to determine the range of knee flexion necessary for tensile testing. Mechanical testing of the anterior cruciate ligament (ACL) measured its viscoelastic properties using an Instron. A 3D model was also created using Mimics (Materialise) for further finite element modelling.

Results: Toluidine blue staining showed changes in the ligaments that were consistent with endochondral ossification, including increased extracellular matrix staining, loss of fibre alignment, and cell hypertrophy near attachment sites. Immunohistochemistry demonstrated modification in collagen type II and sox9 expression in these hypertrophic regions. In the trauma model, collagen type II deposition occurred in the mid-ligament region along with sox9 expression. μCT of the joint space revealed an increase in mineralized tissue volume with increasing severity of OA grades in all models. Gait analysis showed knee flexion between 55 and 100 degrees. Lastly, mechanical testing of the ACL showed the expected viscoelastic behaviour, and elastic modulus was calculated.

Discussion: Overall, ligament pathology is affected during OA progression, and pathology changes are consistent with chondrogenesis and potentially endochondral ossification. The full extent of these changes in the ligament matrix along with the consequences to OA remains to be seen.

Collagen metabolism within a cardiac fibroblasts culture is dependent on physical properties of the cell environment

M. Gałdyszyńska; J. Drobnik

Laboratory of the Connective Tissue Metabolism, Department of Neuropeptides Research, Medical University of Lodz, Poland

Introduction: Extracellular collagen bound by integrins, conveys mechanical forces to the fibroblasts and the signal is supposed to influence metabolism of this protein within the cells. The aim of the study is to assess whether various hardness of the cardiac fibroblasts environment may modify collagen metabolism. Involvement of integrin alfa2beta1 in collagen deposition regulation will be evaluated.

Materials and Methods: The cardiac fibroblasts were cultured on both soft (15 kNt/m^2) or hard (28 kNt/m^2) polyacrylamide gels. Effect of the integrin alfa2beta1 inhibitor—TCI-15 (10^{-7} M, 10^{-8} M)—on collagen deposition was investigated. ITGA2-gene silencing was induced by the use of ITGA2-siRNA. The effectiveness of this process was confirmed by flow cytometry, Western blot and qPCR.

Results: Augmented level of collagen (Woessner method) and expression of type I procollagen alfa 1 chain (qPCR) was found within the culture from the soft gel. Elevated matrix metalloproteinase-1 (MMP-1) and decreased levels of tissue inhibitor of metalloproteinase-3 (TIMP-3) and TIMP-4 were found within fibroblasts cultured on soft gel. Levels of MMP-2, MMP-9, TIMP1, TIMP2 and type III procollagen alfa 1 chain gene expression were not modified. Increased level of mRNA and protein for alfa 2 integrin subunit and alfa2beta1 integrin density was detected in cells from soft gel. Elevation of total and phosphorylated Src kinase content on the fibroblasts from soft gel was observed (immunoenzymatic method). TCI-15 augmented intracellular collagen content in cells cultured on both soft and hard gels.

Discussion: Hardness of environment modify collagen metabolism within cardiac fibroblasts. Thus, investigated physical properties of the fibroblast environment may change type I procollagen alfa 1 chain gene expression and influence collagen catabolism. These processes alter collagen level. Changes of the hardness of the cell environment result in modification of alfa2beta1 integrin receptor density that is accompanied by alterations of Src kinase level and activity. Integrin alfa2beta1 is responsible for inhibition of collagen synthesis.

Supported by grant: OPUS9 nr 2015/17/B/NZ5/01382.

Mechano-regulation of miRNA-221, -222, -21 and -27: implications for articular cartilage homeostasis

P. S. Stadnik*; S. Charlton†; S. J. Gilbert*; J. Tarn†; A. J. Skelton†; M. J. Barter†; V. C. Duance*; D. A. Young†; E. J. Blain*

**Arthritis Research UK Biomechanics and Bioengineering Centre, Biomedicine Division, School of Biosciences, Cardiff University, Cardiff, CF10 3AX; †Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, NE2 4HH*

Introduction : MicroRNAs (miRs) are small non-coding molecules that regulate post-transcriptional target gene expression. Increasing evidence supports a role for epigenetic mechanisms in articular cartilage homeostasis and disease, for example, osteoarthritis (OA). Abnormal mechanical load is a primary risk factor for OA development; previously, miR-221, -222, -146a and -365 were found to be mechano-responsive in chondrocytes. This project aims to (i) examine the mechano-regulation of miRs in articular cartilage subjected to normal and high loads *in vitro*, (ii) validate mechanically regulated miRs in an *in vivo* model of post-traumatic

OA and (iii) identify their downstream targets that could uncouple cartilage homeostasis in OA.

Materials and Methods : Bovine full-depth articular cartilage explants were subjected to loads of 2.5 MPa (normal) or 7 MPa (high) (1 Hz, 15 min) and analysed 24 h post-cessation of load; unloaded explants served as controls. Mechanically regulated miRs were identified using Next Generation Sequencing (The Genome Analysis Centre, Norwich, UK). Expression of identified miRs was quantified in a mouse model of post-traumatic OA (load-induced rupture of the anterior cruciate ligament). Downstream targets of mechanically regulated miRs were verified using 3'-UTR luciferase activity assays.

Results: miR-221 and miR-222, previously shown to be mechano-sensitive, were significantly increased in response to a high (7 MPa) load compared to unloaded explants; both miR-221 and -222 expression also increased with increasing magnitude of load, that is, 7 MPa compared to 2.5 MPa load. miR-21 and miR-27a, known to control genes essential in cartilage homeostasis and regulated in OA, were upregulated in response to 7 MPa load compared to either unloaded or 2.5 MPa load. Interestingly, expression of these miRs was also significantly elevated in the cartilage of the *in vivo* loading model at 7 days post-rupture. *TIMP3* (Tissue Inhibitor of Metalloproteinase 3) and *CPEB3* (Cytoplasmic Polyadenylation Element Binding Protein 3) have been identified as putative downstream targets of these mechanically regulated miRs.

Discussion: Our results confirm miR-221 and -222 mechano-regulation and demonstrate the novel mechano-regulation of miR-21 and -27a in both *in vitro* and *in vivo* loading models, miRs known to be involved in OA. Furthermore, TIMP3 and CPEB3 are putative targets of these differentially regulated miRs and may mediate downstream effects that can lead to alterations in tissue homeostasis and/or cartilage degradation.

Mechanical forces determine the spatial and temporal organisation of essential collagens in the developing skeleton

S. Ahmed; N. C. Nowlan

Department of Bioengineering, Imperial College London, United Kingdom

Introduction: Mechanical forces affect both collagen synthesis and skeletal development. However, the effects of an abnormal mechanical environment on the emergence of collagen organisation have not been described. We examine how abnormal muscle loading affects the structural organisation of collagen type I, II, III, V, VI and X in the developing mouse forelimb.

Materials and Methods: Mice without skeletal muscle were generated ($Pax3^{Spd-/+}$, aka Splotch delayed). Embryos were harvested, staged according to Theiler stages TS22, TS25 and TS27 (typically embryonic days 13.5, 15.5 and 17.5). Collagen distribution was studied with immunofluorescence and confocal microscopy on cryosections.

Results: Abnormal muscle loading affected collagen I distribution at TS22-TS25, with decreased expression in mineralised cartilage compared to controls, but this recovered by TS27. The mesh-like organisation of collagen II of the control cartilage was absent in TS25 mutants. While collagen II was gone from the TS25 control mineralized cartilage, it was still present at low levels in mutants. At TS22, collagen V was expressed throughout the diaphysis of controls but absent in mutants. Collagen V did not appear in mutants until TS25, and even then with milder expression than controls. Collagen VI organisation and fibre orientation, and the shape and height of the chondrons, were abnormal in the mutants between TS25-TS27. Finally, collagen X expression in the mid-diaphysis of the humerus was decreased by at least 15% in the mutants. Collagen III was the only collagen examined that was unaffected by lack of muscle forces.

Discussion: Fetal immobility had dramatic effects on all but one of the collagens examined. The most substantial changes observed were delays in the initial expression of collagen I, II and V, loss of folded structure of the pericellular matrix, reduction in the column length of growth plate chondrons, changes in collagen VI fibre orientation in the mineralised cartilage and reduction in collagen X expression. Mechanoregulation of matrix may be playing a key role in the effects on skeletogenesis when mechanical stimulus is abnormal.

The role of integrin $\alpha_v\beta_3$ in osteocyte mechanotransduction during estrogen deficiency

I. Geoghegan[*,†]; D. A. Hoey[†,‡,§]; L. M. McNamara[*,†]

[*]Mechanobiology and Medical Device Research Group (MMDRG), Biomedical Engineering, National University of Ireland, Galway, Ireland; [†]Centre for Research in Medical Devices (CÚRAM), National University of Ireland, Galway, Ireland; [‡]Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland; [§]Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Ireland

Introduction: The expression of the mechanosensor, integrin $\alpha_v\beta_3$, is reduced in osteoporotic bone cells compared to controls. MLO-Y4 osteocytes experience altered mechanotransduction under estrogen deficiency and it is unknown whether this is associated with defective $\alpha_v\beta_3$ expression or signalling. The objectives of this study are to (1) investigate $\alpha_v\beta_3$ expression and spatial organisation in osteocytes

during estrogen deficiency and (2) establish whether altered responses of osteocytes under estrogen deficiency correlate with defective $\alpha_v\beta_3$ expression and functionality.

Materials and Methods: MLO-Y4 cells were cultured as follows: Ctrl (no added estradiol), E^+ (10 nM 17β-estradiol for 5 days), and Ew (10 nM 17β-estradiol for 3 days and withdrawal for 2 days). Cells were cultured with/without 0.5 μM IntegriSense 750 ($\alpha_v\beta_3$ antagonist). Laminar oscillatory fluid flow of 1 Pa at 0.5 Hz was applied for 1 hr. $\alpha_v\beta_3$ content was quantified using an ELISA. The location and quantity of $\alpha_v\beta_3$ and vinculin (focal-adhesions) was determined by immunocytochemistry.

Results: Estrogen withdrawal under static conditions led to lower cell and focal-adhesion area ($P < 0.05$), compared to E^+ cells. Fluid shear stress led to higher $\alpha_v\beta_3$ content ($P < 0.05$) in all groups, compared to static counterparts, with $\alpha_v\beta_3$ blocking altering this response. Fluid flow on Ew cells had the highest $\alpha_v\beta_3$ levels ($P < 0.05$), but $\alpha_v\beta_3$ did not localise at focal-adhesion sites. Cell morphologies were similar after treatment with the $\alpha_v\beta_3$ antagonist to the Ew group.

Discussion: This study has shown for the first time that cell and focal-adhesion area are reduced following estrogen withdrawal. These results suggest there are fewer functional focal-adhesion sites at which $\alpha_v\beta_3$ integrins localise to facilitate mechanotransduction. To further understand these results, we are analysing osteocyte mechanotransduction by measuring integrin signalling (FAK, p-FAK) and gene expression (COX-2, RANKL, OPG, SOST).

Structure and interactions of elastic fibre proteins ADAMTSL2 and ADAMTSL4

M. Singh; S. A. Cain; A. R. Godwin; M. P. Lockhart-Cairns; A. Roseman; P. Bishop; C. Baldock

Wellcome Trust Centre for Cell Matrix Research, Faculty of Biology, Medicine and Health, University of Manchester United Kingdom

Introduction: Members of A Disintegrin and Metalloprotease with Thrombospondin type 1 motifs-like (ADAMTSL) proteins are extracellular matrix molecules that have been implicated in elastic fibre formation and play a role in fibrillin microfibril assembly and function. Mutations in ADAMTSLs phenotypically mimic genetic disorders caused by mutations in fibrillin-1 indicating that they contribute to common mechanisms in elastic fibre function. ADAMTSL2 interacts with fibrillin-1 and latent TGFβ binding protein-1 (LTBP-1) and mutations in *ADAMTSL2* give rise to geleophysic dysplasia. These data suggest that ADAMTSL2 may contribute to the structural maintenance of microfibrils and play a role in regulating TGFβ bioavailability. Furthermore, ADAMTSL4 has been associated with the structural arrangement of fibrillin-1 microfibrils in ciliary zonules of the eye and mutations in *ADAMTSL4* cause ectopia lentis. However, little is known about the structure and function of ADAMTSLs, except that they lack both the catalytic and disintegrin domains when compared to related ADAMTSs, ruling out a proteolytic function.

Materials and Methods: We have used negative-stain electron microscopy (EM), cryo-EM and small-angle X-ray scattering (SAXS) to determine the structures of ADAMTSL2 and ADAMTSL4. Surface plasmon resonance (SPR) has enabled us to study the interactions of ADAMTSL2 with other matrix proteins. Immunofluorescence microscopy was used to visualise the deposition of ADAMTSL2 and ADAMTSL4 in matrix produced by human dermal fibroblasts (HDFs).

Results and Discussion: Having cloned and purified recombinant ADAMTSL2 from mammalian cell lines, we employed cryo-EM to generate the 3D structure of ADAMTSL2 which revealed an asymmetric lobular shape which was verified by SAXS. SPR showed interactions of ADAMTSL2 with fibronectin and fibrillin-1. Moreover, visualisation of ADAMTSL2 at the cell surface using immunofluorescence microscopy has shown distinct co-localisation with fibronectin, fibrillin-1 and LTBP-1 in HDFs. Structural analysis of ADAMTSL4 using negative-stain EM has revealed that unlike ADAMTSL2, it adopts several conformations suggesting it is highly flexible. We also observed complete co-localisation of the fibrous network of ADAMTSL4 with fibrillin-1 and partial co-localisation with LTBP-1 and fibronectin in HDFs.

Our investigation reports the first structural data on ADAMTSL molecules and furthers our knowledge of their biomolecular interactions providing a better understanding of their function in elastic fibre biology.

Nuclear decoupling is part of a rapid protein-level cellular response to high-intensity mechanical loading

H. T. J. Gilbert[*,†,§]; V. Mallikarjun[*,†,§]; O. Dobre[*,†,§]; M. R. Jackson[*,†,§]; R. Pedley[*,‡,§]; A. P. Gilmore[*,‡,§]; S. M. Richardson[†,§]; J. Swift[*,†,§]

[*]Wellcome Trust Centre for Cell-Matrix Research; [†]Division of Cell Matrix Biology and Regenerative Medicine, School of Biological Sciences; [‡]Division of Molecular and Clinical Cancer Sciences, School of Medical Sciences; [§]Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, M13 9PL, United Kingdom

Introduction: Mesenchymal stem cells (MSCs) have a well-characterised mechano-response, including mechanosensitive commitment to lineage. Cells from mature tissues

must also respond appropriately to the mechanics of their surroundings, with cells in stiff and mechanically stressed environments requiring more robust cellular structures. However, many musculoskeletal disorders and connective tissue pathologies begin at sites of aberrant mechanical loading, suggesting a link between high mechanical stress and musculoskeletal disease. Furthermore, many tissues that experience complex mechanical loads, including heart and muscle tissue, have been proposed to benefit from MSC-based therapies. Whether MSCs have the means to survive and function correctly within high-intensity mechanical strain environments remains unknown. We aimed to understand how molecular processes within MSCs are affected by mechanical stress, and how cells maintain tissue homeostasis in response to elevated mechanical loads.

Materials and Methods: Human MSCs ($n \geq 3$) adhered to type-I collagen-coated silicone membranes were dynamically strained (FlexCell system; low intensity 0-4% strain at 1 Hz and high intensity 2.6-6.2% strain at 5 Hz) for 1 h, followed by a period of recovery. Cells were then analysed using high-content imaging and quantitative -omics technologies (transcript and protein), with and without perturbations to SUN2 expression levels.

Results: At low-intensity strain, morphological changes mimicked responses to increased substrate stiffness. As the strain regime was intensified we characterised rapid establishment of a broad, structured and reversible protein-level response, even as transcription was apparently downregulated. Protein abundance was quantified coincident with changes to protein conformation and posttranscriptional modification. Furthermore, we characterised changes within the linker of nucleo- and cytoskeleton (LINC) complex of proteins that bridges the nuclear envelope, and specifically to levels of SUN domain-containing protein 2 (SUN2).

Discussion: Regulation of the LINC complex decouples mechano-transmission between the cytoskeleton and the nucleus, thus conferring protection to chromatin.

Cellular mechano-environment regulates the mammary circadian clock

N. Yang*,†; J. Williams*,†; V. Pekovic-Vaughan*; P. Wang*,†; S. Olabi*,†; J. McConnell*; N. Gossan*; A. Hughes*; J. Cheung*,†; C. H. Streuli*,†; Q. J. Meng*,†

Faculty of Biology, Medicine and Health, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom; †Wellcome Centre for Cell-Matrix Research, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom

Introduction: Circadian clocks drive ~24-h rhythms in tissue physiology. They rely on transcriptional/translational feedback loops driven by interacting networks of clock complexes. However, little is known about how cell-intrinsic circadian clocks sense and respond to their microenvironment.

Materials and Methods: Time-series microarray analysis was performed using mammary gland tissues isolated from C57BL/6 mice every 4 h over 48 h. Primary mammary epithelial cells were isolated from PER2::LUC reporter mice, and an in vitro 3D culture model was established to investigate the cell-autonomous mammary clock. The mammary stem cell self-renewal capacity was investigated using mammosphere assay in either WT or ClockΔ19 mutant mice.

Results: We identified a functional clock in mammary gland tissue, which rhythmically regulates the expression of ~600 genes with a period of 24 hr. The mammary clock is controlled by the cellular microenvironment in primary cell culture and in vivo. This is consistence with a dampened circadian rhythm observed in aged mammary tissue with stiffened matrix. Mechanistically, the tension sensing cell-matrix adhesion molecule, vinculin, and the Rho/ROCK pathway, which transduces signals provided by extracellular stiffness into cells, regulate the activity of the core circadian clock complex. We also show that genetic perturbation, or age-associated disruption of self-sustained clocks compromises the self-renewal capacity of mammary stem cells.

Discussion: Our work has revealed, for the first time, that circadian clocks are mechano-sensitive. The mammary clock is regulated by cell-matrix interaction, which provides a potential mechanism to explain how ageing influences clock amplitude and function. Furthermore, tissue stiffening suppresses the mammary circadian clock activity in vivo, which could contribute to an increased risk for breast diseases and even cancer.

Collagen crimp in the ageing human optic nerve head

L. Paletto*,†; N. White*,‡; M. P. Fautsch§; V. C. Duance†,¶; J. Albon*,†

Optic Nerve Head Group, School of Optometry and Vision science, Cardiff University, Cardiff, United Kingdom; †Vision Science Bioimaging Laboratories, School of Optometry & Vision Sciences, Cardiff University, Cardiff, United Kingdom; ‡Cardiff Institute of Tissue Engineering & Repair, Cardiff University, Cardiff, United Kingdom; §College of Medicine and Science, Mayo Clinic, Rochester, Minnesota, USA; ¶Pathophysiology and Repair, School of Biosciences, Cardiff University, Cardiff, Wales, United Kingdom

Introduction: Primary open-angle glaucoma (POAG) is the second most common cause of irreversible blindness worldwide. The decreased mechanical compliance of the optic nerve head (ONH) with age may contribute towards the increased susceptibility of the elderly ONH to the development of POAG optic neuropathy. Therefore, this study aimed to quantify ONH collagen crimp as a function of age to investigate its potential role in ONH biomechanics.

Materials and Methods: Image datasets acquired from an age range of human ONHs ($n = 9$, aged 0-30 years, 31-60 years and 61-88 years) and glaucomatous ($n = 3$) cryosections were excited at 800 nm/12 and forward-scattered second harmonic generation (SHG) signals were collected using a wavelength ultra-fast tuneable 140 fs mode-locked laser scanning microscope (LSM880 NLO with AxioExaminer™ stand; Carl Zeiss Ltd, UK). Crimp period (CP), crimp amplitude (C_{Amp}) and degree of crimp were quantified in image datasets acquired from the lamina cribrosa (LC) and peripapillary sclera (ppsclera).

Results: Fibrillar collagen bundles in OHN from individuals <10 years of age had a wavy appearance which differs from that observed in older ONH (above the age of 40 years). However, C_{Amp} in ppsclera ($P = 0.122$) and LC ($P = 0.444$) and degree of crimp in ppsclera ($P = 0.170$) and LC ($P = 0.546$) did not change with age. Ppsclera CP increased significantly from 19.64 μm and 18.54 μm in the 2- and 6-year-old, respectively, to 22.04 μm in the 88-year-old (rs=0.850, $P = 0.004$). Additionally, LC CP was significantly greater in two of the three glaucoma ONHs, compared to age-matched controls ($P = 0.000$). No differences were found in the C_{Amp} ($P = 0.139$) and degree of crimp ($P = 0.723$) in the glaucomatous LC and ppsclera.

Discussion: The changes in collagen crimp parameters within the ppsclera and LC as a function of age and/or glaucoma will influence ONH mechanics in response to intraocular pressure (IOP) elevation, and thereby effect mechano-transduction of cells within the LC.

Perineuronal chondroitin sulfates regulate excitation/Inhibition balance in developing vestibular circuitry

T. P. Y. Kwan*; K. L. K. Wu*; D. K. Y. Shum*,†; Y. S. Chan*,†

*School of Biomedical Sciences, The University of Hong Kong, Sassoon Road, Hong Kong, China; †Research Centre of Heart, Brain, Hormone and Healthy Aging, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Sassoon Road, Hong Kong, China

Introduction: Perineuronal nets (PN) are implicated in restricting neural plasticity with the maturation of neural circuits. Our study of the central vestibular nucleus (VN) found consolidation of PN around GABAergic interneurons as from postnatal day (P) 9 of Sprague Dawley (SD) rats. Cleavage of chondroitin sulfates (CS) chains by microinjection of chondroitinase ABC (ChABC) resulted in delay of PN formation from P9 to P13. We hypothesized the delay would affect the excitation/inhibition balance in developing vestibular circuitry and acquisition of vestibular-dependent behaviours.

Materials and Methods: To find if cleavage of PN-CS by ChABC injection impacts on inhibition and excitation circuitry in VN during development, whole-cell patch-clamp recordings of spontaneous inhibitory and excitatory post-synaptic current (sIPSC and sEPSC) from VN interneurons were performed in brainstem slice preparations of P9 and P14 rats following test treatment with ChABC in the VN of P6 rats.

To find if PN-CS and PN-associated Semaphorin 3A bears behavioural consequences, the rats were assessed for developmental emergence of air-righting as read-outs of the maturation of the circuit for graviception.

Results: Significant reduction of sIPSC frequency and enhancement of sEPSC amplitude indicated disruption of PN-CS interfere the balance of excitation and inhibition in the circuitry.

Progressive localization of plasticity-inducing factor, Sema3A, to PN-CS was observed from P9 in VN. ChABC/Sema3A treatment of the rat VN at P6 resulted in delayed display of air-righting.

Discussion: CS disruption triggered plasticity at excitatory and inhibitory synapses onto otherwise PN-enwrapped neurons in the VN circuit. The behavioural readouts provide evidence for the role of PN-CS-Sema3A in controlling structural and circuit plasticity at the interneuron level with impacts on the developmental display of graviceptive behaviour.

Acknowledgement: The project is supported by general research fund (RGC-GRF 17125115).

Cell mechanical response to growth factors and cross-linking in human engineered tendon constructs

A. Giannopoulos; R. B. Svensson; C.-Y. C. Yeung; M. Kjaer; S. Peter Magnusson

Institute of Sports Medicine Copenhagen, Bispebjerg-Frederiksberg Hospital, University of Copenhagen, Denmark

Introduction: Tendons transmit forces and maintain an internal tension that is a reciprocal relation of cell-generated forces and matrix properties. The mechanical forces are dependent on tissue properties and can be regulated by growth factors. The purpose of this study was to characterize the functional properties of human engineered constructs in defined mechanical alterations.

Materials and Methods: Human tendon fibroblasts within a fibrin scaffold gradually created their own matrix, which structurally resembles *in vivo* tissue. We manipulated matrix stiffness by a natural cross linker, genipin and also compared force responses with IGF-1 and TGF-b treatment. A custom-made force monitor was used which can

apply force in a stepped manner and measure the outcome force at the same time, in culture conditions. The engineered tissues were subjected to cyclic unloading-loading protocols.

Results: When unloading the construct, cell re-tension was observed during the rest period, which is a tensional homeostatic response. Following a loading step, matrix relaxation was measured, which is due to viscoelastic properties of the constructs. The cell re-tension of genipin treated constructs was reduced in a dose-dependent manner while the stress relaxation followed the same pattern. In contrast, genipin enhanced the peak modulus only at the highest concentration and the stiffness only at medium and high concentrations. BAPN treatment used as a negative control to prevent cross-link formation and confirmed by reducing dramatically the stiffness and the peak modulus whereas it had no effect on cell re-tension and matrix relaxation. Treatment with IGF-1 did not alter functional properties of the constructs whereas with TGF-b addition the cell re-tension increased compared with the untreated controls and IGF-1 treated. The matrix relaxation was reduced only by TGF-b treatment.

Discussion: Disruption of tensional homeostasis eventually can lead to pathological conditions. These data provide useful information that may lead to specific targets regarding tissue healing or regeneration.

The synovial surface of the articular cartilage. Ultrastructural aspects

C. Dell'Orbo; M. Reguzzoni; M. Protasoni; M. Raspanti

Laboratory of Human Morphology, Insubria University, Varese, Italy

Introduction: Articular cartilage has been for long time the subject of intense research because of its evident clinical relevance. The composition and architecture of its matrix have been deeply investigated while its articular (synovial) surface, by contrast, has been the subject of few and sparse studies even it is obviously the only portion directly involved in the articular function. The present research represents a preliminary approach to an ultrastructural study on the articular surface.

Materials and Methods: Fragments of bovine articular cartilage were observed either unfixed by Fluid TM-AFM or briefly fixed in 1% Karnowski, dehydrated in graded ethanol and Hexamethyldisilazane (HMDS) and observed by SEM. Other fragments were fixed in Cupromeronic Blue (CB) or in Ruthenium Hexamine Trihydrate (RHT), dehydrated and observed by SEM. Finally, other samples were fixed overnight in 1% Karnowski, treated for 5 days with 2N NaOH and dehydrated and observed as above.

Results: In all cases the articular surface was made of thin, parallel fibrils immersed in a great amount of glycoconjugates, as confirmed by Fluid TM-AFM, CB and RHT. Treatment with concentrated NaOH completely removed all non-collagenic material and revealed a continuous surface of very thin (approx. 15 nm), uniform fibrils with various orientations depending from the sampling site. There were occasional evidences of a layered arrangement. Neither chondrocytes nor empty lacunae were detectable on the articular surface. The subsurface matrix down to the subchondral bone exhibited sparse normal chondrocytic lacunae and larger, heterogeneous collagen fibrils with diameters up to 150 nm.

Discussion: The free (synovial) surface of the articular cartilage is devoid of cells and composed of a uniform population of very thin, very regular collagen fibrils interconnected by copious proteoglycans. The fibrils are similar to those of the pericellular matrix and quite different from the larger, heterogeneous fibrils of the interterritorial matrix. It can be hypothesized that the articular surface is a sort of immature layer continuously deposed from the underlying chondrocytes and that it can have functional properties different from the subsurface matrix. The research is still underway.